Effects of Blood Contamination on Cerebrospinal Fluid Cell Counts, Protein, and D-dimer Concentrations

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(Abstract)

Cerebrospinal fluid analysis (CSF) is commonly performed in clinical neurology, and is a sensitive, but non-specific indicator of central nervous system (CNS) pathology.
Blood contaminated CSF samples have the potential to adversely affect results of cytologic, serologic, microbiologic, and molecular biologic diagnostics. A clear consensus of the effects of blood contamination on CSF analysis could not be drawn following a review of the existing veterinary literature. Based on data from earlier reports, it was hypothesized that iatrogenic blood contamination of CSF would result in significant increases in both the CSF total protein (TP) concentration and nucleated cell count (WBC). As hypothesized, *in vitro* CSF blood contamination resulted in statistically significant ($p < 0.01$) linear increases in both the CSF TP and WBC with increasing RBC concentration in CSF from sixteen normal dogs. Although increases in TP and WBC are statistically significant, their clinical impact is negligible. Results of this study demonstrate that in normal dogs, the mean CSF TP concentration collected from the cerebellomedullary cistern, is lower than previously reported.

D-dimers are plasminolytic cleavage products formed by the cross-linkage of fibrin by Factor XIIIa. In humans, D-dimer analysis can be used to differentiate iatrogenic from pathologic CNS hemorrhage. An additional objective of this study was to determine if canine D-dimers could be assayed using commercially available latex agglutination (LA) and enzymatic immunoassay (EIA) kits in normal and diseased subjects. It was hypothesized that qualitative and quantitative determinations of blood and CSF D-dimer activities could be aid in the diagnosis of dogs with altered CNS and/or systemic coagulation.

D-dimers were able to be assayed in all subjects studied. D-dimer concentrations in CSF samples, when analyzed using a qualitative LA assay system, from healthy dogs with iatrogenically blood contaminated CSF were consistently negative. Quantitation of CSF D-dimer concentrations in normal dogs using an EIA assay resulted in lower values (mean 16.2 ± 4.3 ng/ml; range, 0 to 54 ng/ml) than detected in the peripheral blood of dogs and humans (normal cutoff value < 250 ng/ml). These findings suggest that D-dimer formation does not occur in canine CSF freshly contaminated with blood.

Significantly ($p < 0.001$) higher mean blood D-dimer concentrations were present in dogs with systemic coagulation disorders ($1{,}093.4 \pm 172.3$ ng/ml; range, 0 to > 2,000 ng/ml) when compared to normal dogs (54.6 ± 19.8 ng/ml; range, 0 to 190 ng/ml), when assayed with the EIA. When used as an adjunct in the diagnosis of systemic coagulation abnormalities, the EIA assay had an overall sensitivity of 92%, specificity of 100%, positive predictive value (PPV) of 100%, and negative predictive value (NPV) of 94%. When applied to the same dogs, the LA D-dimer was less sensitive and specific (sensitivity of 73%, specificity of 100%, PPV of 100%, and NPV of 80%) than the EIA.

Evidence of intrathecal fibrinolysis in the absence of systemic abnormalities was also demonstrated using CSF LA and EIA D-dimer assays in some dogs with a variety of infectious (Rocky mountain spotted fever), non-infectious inflammatory (granulomatous meningoencephalitis, steroid-responsive meningitis), traumatic (intervertebral disc disease, spinal fracture), and neoplastic (meningioma) diseases. When all dogs with CNS diseases were examined together, the mean EIA D-dimer concentration was significantly ($p = 0.03$) higher (511.6 ± 279.8 ng/ml) than normal dogs (mean 16.2 ± 4.3 ng/ml). Future studies will be required before the definitive role of D-dimer analysis can be defined in veterinary medicine.

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Multimedia Object Listing

Alphabetical Key of Abbreviations

Abbreviation	Translation
ACTH	Corticotropin hormone
AIHA	Autoimmune hemolytic anemia
aPTT	Activated partial thromboplastin time
AQ	Albumin quotient
ATIII	Antithrombin III
BBB	Blood-brain barrier
BG	Blood glucose concentration
BUN	Blood urea nitrogen
CBC	Complete blood count
CMC	Cerebellomedullary cistern
CNS	Central nervous system
CSF	Cerebrospinal fluid
CT	Computed tomography
DIC	Disseminated intravascular coagulation
DM	Degenerative myelopathy
DVT	Deep venous thrombosis
EIA	Enzymatic D-dimer immunoassay
FDP	Fibrin degradation products
GME	Granulomatous meningoencephalitis
GSD	German Shepherd dog
HAC	Hyperadrenocorticism
Hg	Mercury
HSA	Hemangiosarcoma
ICP	Intracranial pressure
IL-6	Interleukin 6
IM	Intramuscular
IV	Intravenous
IVDD	Intervertebral disc disease

Abbreviation	Translation
LA	Latex agglutination D-dimer assay
MRI	Magnetic resonance imaging
NPV	Negative predictive value
OSPT	One-step prothrombin time
paO2	Arterial partial pressure of oxygen
PBS	Phosphate buffered saline
PCV	Packed cell volume
PPV	Positive predictive value
PTE	Pulmonary thromboembolism
PU/PD	Polyuria/polydipsia
RBC	Red blood cell concentration
RMSF	Rocky mountain spotted fever
SRM	Steroid responsive meningitis
TP	Total protein concentration
TNCC	Total nucleated cell count = WBC
TNF-α	Tumor necrosis factor alpha
UP:UC	Urine protein: creatinine ratio
WBC	White blood cell concentration

Chapter 1- Introduction and Literature Review

Introduction

Cerebrospinal fluid (CSF) analysis has an important clinical role in the identification of central nervous system disease, and may provide specific diagnostic, therapeutic, and prognostic information. Routine laboratory examinations performed on CSF in veterinary medicine include specific gravity, total protein (TP) and glucose concentrations, red blood cell (RBC) and white blood cell (WBC) counts, leukocyte differential counts, quantitative microbial culture, and cytologic examination.[1-3] Serology, protein electrophoresis, immunocytochemistry, molecular tests (polymerase chain reaction), and various biochemical enzymes can be evaluated, but are not considered part of routine CSF evaluation. The availability, methodology, and interpretation of tests performed on CSF have been previously described.[1-13]

A commonly encountered problem in the analysis of CSF is its iatrogenic contamination with peripheral blood during sample procurement. The introduction of blood into CSF presents diagnostic challenges to both the clinician and pathologist, as blood contamination can potentially alter RBC and WBC counts, total protein concentration, specific protein fractions, and biochemical, microbiologic, and serologic tests.[1-6,11-13] In veterinary medicine, few attempts have been made to describe the effects of blood contamination on CSF interpretation.[5,11,12] Additionally, some methods employed in both human and veterinary medicine to "correct" for iatrogenic blood contamination have been questioned.[11-13] The following study reviews the effects of peripheral blood contamination on the laboratory evaluation, particularly the protein concentration, and clinical interpretation of CSF.

Literature Review

1) CSF Function, Production, Absorption, and Composition

Cerebrospinal fluid is located in the subarachnoid space and serves as a mechanical cushion for central nervous system (CNS) tissue. It also regulates CNS homeostasis by collecting cellular waste products, supplying and circulating nutrients, immunoglobulins, cytokines, and other factors. Using a variety of techniques such as

radionucleide measurements, free fluid drainage studies, and inulin dilution/clearances, CSF formation and fluid dynamics has been studied extensively in a variety of species.[8]

Cerebrospinal fluid production occurs primarily in the choroid plexus of the ventricular system by ultrafiltration of plasma and subsequent active secretion. In canine studies the ventricular choroidal system produces up to 57% of the total CSF, with the remainder being formed by ventricular ependymal cells, and possibly by the cerebral subarachnoid endothelium.[8] Experimental choroid plexectomy of the lateral ventricles in dogs resulted in significant drops in the daily rate of CSF production, confirming the importance of the ventricular choroidal plexus in CSF production. In the dog and cat, the daily rate of CSF production has been reported as 0.047 ml/min and 0.016 ml/min, respectively.[8]

Tight junctions between capillary endothelial cells and the choroid plexus epithelium restrict passage of large macromolecules from the blood into the CNS and constitute the anatomic blood-brain barrier (BBB). A physiologically dynamic BBB also exists, which is defined as the steady state equilibrium between serum and CSF as quantitated by protein concentration ratios of the two compartments.[3,8] The pathophysiologic mechanisms ultimately responsible for abnormalities in CSF result in a disruption of the anatomic or physiologic blood-brain barriers, aberrations in the intrathecal microenvironment (intact blood-brain barrier), or combinations of both.

Arachnoid villi are responsible for resorption of CSF into the vascular system, as indicated by autoradiographic and fluorescein-labeled protein studies.[6] As CSF pressure is normally greater than blood pressure in the dural sinuses, a hydrostatic gradient exists which favors escape of CSF into blood. Early evidence suggested that CSF secreted into the ventricles flowed out of the cisterna magna and was later absorbed by arachnoid villi by the serosinuses.[6,8] Flow of CSF is driven by the unvalved pumping action of the choroid plexuses. However, other studies evaluating CSF flow patterns have produced conflicting results, and further study is required to precisely map flow of CSF.[3,4,6]

Although CSF is derived by filtration of plasma, its biochemical composition is not identical to that of plasma (Appendix A).[6] Active transport between CSF, interstitial brain fluid, neuroglia, and intracranial capillaries continually modifies the constituents of the ultrafiltrate of plasma that is produced in the choroid plexus.[6,8] It has also been

demonstrated that the biochemical nature of CSF differs between different anatomic areas of the CNS.[5,7,12]

2) Indications for and Contraindications of CSF Analysis

There are multiple indications for CSF collection and analysis, especially in cases of clinically suspected CNS inflammatory disease. Serially performed CSF analyses are valuable in assessment of therapeutic efficacy in certain infectious and inflammatory meningoencephalitides, especially when used in conjunction with serologic techniques.[3,12] Pathophysiologically, the disease process must involve or have extension into the subarachnoid space or the ventricular system to produce cellular abnormalities that can be detected with routine CSF analysis.[2] Although CSF collection is reasonably safe, there are several notable contraindications, such as severe systemic or neurologic compromise, and an increased intracranial pressure (ICP), as brain herniation may result from rapid pressure changes associated with CSF collection. The performance of advanced intracranial imaging procedures prior to the performance of CSF collection has been recommended to facilitate the identification of pathologic processes that may be associated with increases in ICP, and identify patients at increased risk for brain herniation.[1-3]

3) Review of Routine CSF Evaluation and the Effects of Blood Contamination on CSF Analysis

The first step in analysis of CSF is specimen collection. Cerebromedullary cisternal or lumbar punctures are routinely performed in veterinary medicine. Procedures for obtaining CSF, and differences in protein concentration and cellular composition between these two sites of collection have been reviewed.[2,7] Once a specimen is obtained, routine evaluation of CSF can be subdivided into five categories: gross examination, cellular counts, cytological examination, biochemical testing, and microbiologic studies. Peripheral blood contamination can confound CSF interpretation during any step of the routine evaluation. Contamination of CSF with peripheral blood is not uncommon because of the "blind" manner in which samples are obtained. However, the risk of peripheral blood contamination in veterinary medicine may be higher because

of greater anatomic variations between species and breeds, which makes sample procurement difficult, even in the hands of skilled clinicians.

4) Gross Examination

Gross visual inspection of the CSF specimen is typically performed immediately after collection. Normal CSF is clear and colorless. Various disease states result in cloudy, turbid, bloody, clotted, viscous, or discolored fluid. [1-3,15] Turbidity and cloudiness are often due to pleocytosis (leukocyte count >200/µl), the presence of microorganisms, excessive RBC's (>400/µl), or an increased protein concentration. Pink or red CSF usually indicates the presence of blood, but gross inspection cannot differentiate between subarachnoid hemorrhage, intracerebral hemorrhage, infarcts, or a traumatic CSF tap. It is extremely important to differentiate pathologic processes from iatrogenic trauma, and a number of procedures have been devised to help distinguish a traumatic tap from other causes of hemorrhage.

During CSF collection, traumatic punctures tend to result in the maximum amount of blood being detected in the early volume of fluid, with a subsequent decrease in hemorrhage as collection volume increases. Centrifugation of CSF results in a clear supernatant in cases of iatrogenic trauma, and xanthochromia (pink/yellow discoloration due to hemoglobin breakdown) with other causes of hemorrhage.[1,4,5,11] However, acute pathologic causes of CNS hemorrhage may result in a clear supernatant following centrifugation up to several hours following injury. Therefore, the finding of a clear supernatant does not definitively rule out acute CNS bleeding or a traumatic tap, with or without concomitant acute pathologic CNS hemorrhage. It is important that CSF be evaluated within 30 minutes of collection because lysis of RBC's begins within one hour, and can result in false-positive detection of xanthochromia.[1-3] Xanthochromia may also result from jaundice, protein concentrations exceeding 150 mg/dl, hypercarotinemia, and certain intrathecal neoplasms (particularly melanoma).[11] The use of spectrophotometry to objectively record xanthochromia has been advocated, but visual inspection has been shown to have comparable sensitivity.[2,15] A variety of other gross abnormalities of CSF may exist: greenish tinges may be seen in cases of purulent CSF fluid due to elevated

myeloperoxidase, and fat globules may be visualized secondary to fat embolization or aspiration of adipose tissue during sample collection.[6]

5) Cell Counts

Cell counts are performed once gross examination has been completed. Analysis of CSF should take place within 30 minutes to an hour after collection to avoid cellular lysis due to the hypotonic nature of CSF.[1,2] Recently, the addition of autologous serum to CSF allows for accurate cytological analysis for up to 48 hours following collection in dogs and cats.[16] The low cellularity of normal CSF (0-8 leukocytes/μl) precludes the use of automated cell counters in CSF evaluation. Thus, a hemocytometer chamber is used to determine cell counts. Red cells are not normally present in cerebrospinal fluid. The presence of red blood cells in CSF can significantly alter the total nucleated cell count as a result of the influx of RBC's and WBC's associated with hemorrhage, regardless of etiology. In cases where a traumatic tap is suspected, a count of the RBC's in the CSF may be used to correct CSF leukocyte counts. This method assumes that all RBC's present are due to traumatic damage and not to pathologic hemorrhage, and will approximate the true CSF leukocyte count because of the imprecision of the CSF RBC count. The formula for the correction is as follows: [2,13,14]

$$W = WBC_f - \frac{WBC_b \times RBC_f}{RBC_b}$$

W= CSF leukocyte count before blood was added

WBC_f = total CSF leukocyte count

WBC_b = blood leukocyte count

RBC_f = total CSF RBC count

RBC_b = RBC count blood

In normal animals, this correction formula accounts for approximately 1-2 leukocytes/1,000 RBC's. Several studies have questioned the validity of this formula, and cite that this accepted formula tends to overcorrect WBC counts, and thus obscure

possible infectious processes.[2-4,17,18] However, controlled studies investigating the reliability of correction formulas in veterinary medicine are lacking.

The site of CSF collection also affects the total nucleated cell count. In normal dogs there is a significant difference in total number of WBC's present in CSF samples obtained from the cerebellomedullary cistern (CMC) and lumbar areas, with the CMC counts being higher.[2,5,7] The location of sampling must be taken into consideration when analyzing CSF samples.

6) Cytologic Examination

The predominant cells of the differential count of normal CSF are lymphocytes and macrophages. An occasional non-degenerate mature neutrophil and ependymal cell can be a normal finding in dogs. In one human study, the number of neutrophils in CSF was strongly correlated to the degree of blood contamination in the sample and to the patient's peripheral neutrophil count.[14] Extrapolating from studies in human medicine, and applying the correction formula, it is accepted in veterinary medicine that blood contamination of the CSF samples will result in increased numbers of neutrophils in the differential count. Additional cells that may be seen in normal CSF, especially associated with traumatic taps include bone marrow cells, chondrocytes, squamous epithelial cells, myocytes, and fibroblasts. Bone marrow cells may be obtained in specimens that are difficult to obtain, or when specimens are obtained from patients with vertebral body abnormalities. Chondrocytes may be seen when the spinal needle inadvertantly contacts the intervertebral disc. Squamous cells, myocytes, and fibroblasts are artifacts from the penetration of the needle through soft tissues overlying the subarachnoid space. Effects of blood contamination on the interpretation of CSF cytology stem from the detection of abnormal cells or organisms that may have originated in the blood or CNS. An example is blood contamination of CSF resulting in the misdiagnosis of CNS leukemia in the event of peripheral lymphoblasts being introduced into the sample. Bone marrow cells have also been interpreted as neoplastic when observed in CSF.[2]

7) Microbiologic Analysis

Microbiologic analysis of CSF is important in cases of suspected infectious encephalitis or meningitis. Bacterial, fungal, protozoal, and viral central nervous infections are well documented in the veterinary literature.[1,6,7,10] Although the frequency of infectious meningoencephalitis is low in companion animals, microbiologic analysis is frequently indicated in veterinary patients. The potential of false-positive bacterial and viral CSF cultures because of contamination with peripheral blood has been documented in humans, but has not been investigated in animals. However, as dogs and cats are frequently diagnosed with septicemia, the possibility of obtaining a positive CSF culture because of CNS contamination with infected peripheral blood is not implausible. Somewhat related is the presence of intact bacteria in CSF cytology specimens that result from inadvertant introduction of skin flora to the sample during collection, even when strict aseptic technique is utilized.[3]

8) Laboratory Tests

The remaining procedures performed on CSF are clinicopathologic laboratory tests. Tests commonly performed include: specific gravity, glucose determination, and protein concentration. Normal canine CSF specific gravity ranges between 1.004-1.006, as measured by temperature compensated refractometry. A retrospective study performed in dogs concluded that determination of CSF specific gravity does not provide significant diagnostic or prognostic information, and as a result, has lowered the use of CSF specific gravity in the clinical setting.[18]

Determination of CSF glucose concentration is also commonplace. CNS glucose concentration is dependent on blood glucose levels. Glucose enters the CSF by facilitated diffusion in the choroid plexus. Because CNS glucose concentration is diffusion dependent, changes in plasma glucose levels may take several hours to be reflected in the CNS. It is therefore recommended that plasma glucose be obtained at the time of CSF collection. Normal CSF glucose concentration is 60-80% of plasma glucose levels.[2,3] Elevated CNS glucose concentrations confirms the presence of previous or concurrent hyperglycemia, and may be seen with iatrogenically blood contaminated CSF

specimens. Hyperglycorrachia is a non-specific finding that is important when dealing with diabetic patients. Diagnostically, hypoglycorrhachia is more significant and is seen in cases of bacterial or fungal meningitis, symptomatic hypoglycemia, primary or metastatic neoplasms, and subarachnoid hemorrhage. Hypoglycorrachia seen with subarachnoid hemorrhage is due to the release of glycolytic enzymes by erythrocytes, and may serve as an additional way of differentiating pathologic hemorrhage from iatrogenic blood contamination.[3] Previously, it was described that hypoglycorrachia in cases of infectious meningitis were due to glucose utilization by microorganisms and leukocytes present in the CNS.[3,19] It is unlikely these mechanisms are responsible for the decreased glucose concentrations, as the low numbers of microorganisms observed in infectious meningitis and the small amount of glucose utilized by invading leukocytes is insufficient to explain the degree of hypoglycorrachia present. It is hypothesized that defective glucose transport and compensatory CNS anaerobic glycloysis are responsible for the decreased CSF glucose concentrations seen in cases of viral, fungal, and bacterial meningitis. The low CSF glucose level in these cases is often accompanied by an increase in the lactate concentration.[3,19]

The final chemical analysis routinely performed on CSF is the protein concentration. Normal CSF contains less than 1% of the protein concentration found in plasma. Most CSF protein is derived from plasma, and the presence of prealbumin, transferrin, and nervous tissue specific proteins in CSF accounts for most of the qualitative differences between CSF and plasma proteins. The hydrodynamic radius of plasma proteins is the major factor that regulates protein diffusion into the CNS, not the molecular weight.[8] Some areas of choroid plexus epithelium deliver large proteins to the CNS by pinocytosis.

CSF protein concentration varies by site of collection and ranges from 10-25 mg/dl from the CMC, and <45 mg/dl from the lumbar region.[7] CSF protein can be measured qualitatively or quantitatively. Qualitative measurements are more easily performed than quantitative procedures, and are often done while waiting for quantitative laboratory results. Urinary reagent strips provide a rough estimate of increased CSF protein (>100 mg/dl), but are insensitive in the detection of protein levels less than 100

mg/dl.[2] Pandy's and the Nonne-Apelt tests are also utilized for the qualitative identification of increased CSF globulins. These tests are based upon turbidity changes that occur in the presence of an increased CSF globulin fraction.[2,3,7]

Analytical methods for quantitative CSF total protein determination are more difficult than other body fluids because of the low protein concentration in CSF, the small volume of fluid available for analysis, presence of many different types of proteins that subsequently react differently with available reagents, and the wide variation in methods described.[1,2,4,6,8-10,20] The most common methods utilized for protein determination are turbidimetric procedures using trichloroacetic acid or sulfosalicylic acid reagents. These methods are rapid and easily performed with readily available laboratory instrumentation. However, the major drawbacks with turbidimetric analysis are that albumin produces more turbidity than comparable amounts of globulins, and that strict temperature control of the assay must occur, because turbidity is linearly related to temperature.[20] Colorimetric techniques are also employed in the determination of CSF protein. The Lowry method is described as being highly sensitive and reasonably specific, and is often used as the standard of comparison for CSF protein determination.[9,10,14] The Lowry technique is least affected by albumin:globulin ratios when compared to other methods, but is labor intensive and can be influenced by non-protein CSF components. The Coomassie brilliant blue and Pyrogallol red colorimetric assays are reliable and highly sensitive if properly standardized, and are more rapid than the Lowry test.

Other methods, such as spectrophotometry, immunologic measurements, dye binding, and biuret tests, have been advocated to measure CSF total protein concentration, each with its own advantages and disadvantages. However, studies evaluating the variety of methodologies available for CSF protein determination concluded that choice of standardization was more important than the method used to analyze CSF protein.[21,22] As a result, the Coomassie brilliant blue, trichloroacetic acid, and biuret methods have all been accepted as methods for quantification of CSF total protein.

Elevation in CSF total protein is the most common laboratory abnormality noted on CSF examination.[1-3] Although this finding is non-specific, its presence indicates central nervous system disease, which warrants further investigation. Elevated CSF

protein is often seen with pleocytosis, but can occur as an isolated abnormality, in which case it is termed albuminocytologic disassociation. Because of the previously described anatomic and physiologic blood-brain barriers, elevations in CSF protein concentrations can be explained by several mechanisms: a breech in the blood-brain barrier, local (CNS) production of protein (immunoglobulin), degeneration of nervous tissue, or blockage of normal CSF flow or absorption.[1,2] As CSF albumin is derived exclusively from plasma, increased CSF albumin may be used to assess the integrity of the BBB by determining the serum: CSF ratio of albumin (albumin quotient, AQ).[1-3] The AQ is calculated as follows:

$$AQ = \frac{\text{CSF albumin (mg/dl)}}{\text{Serum albumin (g/dl)} \times 10}$$

Normal AQ in dogs ranges from 0.17 to 0.3, and elevations in the AQ can be explained by loss of BBB integrity.[2] Direct measurement of CSF albumin concentration can be performed using rocket immunoelectrophoresis, which requires small volumes of cerebrospinal fluid.

The AQ can be low, normal, or increased in cases of CNS inflammatory, degenerative, or neoplastic diseases. Several studies describing the effects of CNS diseases on the AQ are available.[1-3,6,16] The AQ provides the most information when interpreted in conjunction with the IgG index, total protein concentration, cell counts, and cytologic findings. To date, immunoglobulins have been identified as the only diagnostically significant proteins locally synthesized in the central nervous system.[3,6,9] Thus, it is clinically important to distinguish locally produced immunoglobulin from immunoglobulins derived from passive serum transfer into the CNS. To accomplish this task, a parameter must be developed and analyzed that reliably evaluates the integrity of the blood-brain barrier. The predominant CSF immunoglobulin in dogs and cats is IgG, which can be found in small amounts in CSF. However, CSF IgG levels vary between species, breeds, individuals, and may be partially dependent on serum IgG concentrations. Reference IgM and IgA concentrations are negligible in companion animal CSF.[2,3]

Limits of serum-CSF concentration ratios have been described in the human literature for the passive transfer of immunoglobulins in normal and pathologic states.[6] This study used a disc electrophoretic protein assay to evaluate blood-brain barrier integrity in a variety of physiologic and pathologic states, and was shown to be reliable except in cases of acute inflammatory disease.[6] In veterinary medicine, the IgG index is utilized to determine if increases in CSF immunoglobulins are due to local synthesis or alterations in the BBB. The IgG index is calculated by first determining the ratio of CSF to serum immunoglobulins as follows:

$$\text{IgG ratio} = \frac{\text{CSF IgG}}{\text{Serum IgG}}$$

Quantification of CSF immunoglobulins using radial immunodiffusion (RID) or immunoelectrophoretic procedures has been described.[9,20] As disruption of the BBB can produce alterations in the IgG ratio, consideration of the IgG ratio in relation to the AQ is necessary to accurately assess the degree of local humoral production versus influx of serum immunolglobulin due to BBB pathology. This relationship is defined as the IgG index:

$$\text{IgG Index} = \frac{\text{IgG Ratio}}{\text{AQ}}$$

IgG index values greater than 0.9 are considered indicative of intrathecal IgG synthesis.[2] Calculation of the AQ and the IgG index, coupled with CSF protein electrophoretic procedures provides ancillary diagnostic information to the clinician. The effects of CNS inflammatory, degenerative, traumatic, and neoplastic disease processes on the AQ, IgG index, and interpretation of protein electrophoresis have previously been described.[1-3,5,6,10]

The role of peripheral blood contamination of CSF samples is also pivotal in the quantification of CSF total and specific protein concentrations. Theoretically, it is

proposed that because peripheral blood contains several thousand milligrams of protein/dl and CSF contains tens of milligrams of protein/dl, blood contamination should have a significant effect on CSF total protein concentration. In cases of suspected peripheral blood contamination, a formula has been devised to correct for the degree of protein elevation due to blood contamination, and is similar to the formula described for correction of the WBC count. The formula utilized for the correction of the CSF protein is as follows: [3,17]

$$\text{mg/dl added protein} = \frac{[\text{serum protein (mg/dl)} \times (1.00 - \text{Hct})] \times \text{CSF RBC's/}\mu\text{l}}{\text{peripheral blood RBC's/}\mu\text{l}}$$

It is important when using this correction formula that cell counts and protein determination be performed on the same CSF sample tube. When applying this formula, approximately 1 mg/dl of protein can be subtracted from the CSF total protein measurement per 1,200 RBC's present.[1] Although peripheral blood contamination significantly alters CSF protein determinations, two studies have shown that CSF protein concentration is not critically affected by moderate degrees of hemorrhage up to 14,000 RBC/µl.[11,12] These uncontrolled studies were performed across several species, and involved clinically normal and diseased subjects. Results from one earlier veterinary report questioned the validity of the previously mentioned formula when used for correction of the CSF total protein concentration in the face of blood contamination, and stated that the effect of blood contamination on the protein concentration was unclear.[17] However, this study lacked strict controls.[17]

9) Ancillary CSF Laboratory Testing: D-dimers

A wide spectrum of coagulation abnormalities have been described in veterinary and human clinical medicine.[23-45] In humans, these hemostatic disorders are major contributors to patient morbidity and mortality, prolonged hospitalization, and increased health care costs. Recently, efforts have been made in diagnostic medicine to identify and validate a rapid, reliable, and easily performed adjunctive clincopathologic tests that facilitate early recognition and characterization of hemostatic dysfunction. Of the tests

that have been developed for this purpose, D-dimer assays are among the most commonly used in clinical medicine, although the diagnostic utility of D-dimers has not been firmly established. [46-66]

The detection of fibrinogen degradation products (FDP) in conjunction with other clinicopathologic tests has been widely used for the diagnosis of thrombogenic conditions.[25] Because FDP detection systems utilize non-specific polyclonal antibodies against fibrin or fibrinogen derivatives, FDP determination fails to distinguish between fibrin and fibrinogen degradation products, and cannot be measured using plasma samples. Because of these deficiencies, coagulation research was conducted to identify specific derivatives of fibrin in the blood of people with various coagulopathies. The end result of these studies were the identification of specific derivatives of cross-linked fibrin, D-dimers.[25,28,33,49,53]

D-dimers are plasminolytic cleavage products formed by the cross-linkage of fibrin by Factor XIIIa (Figures 1.1 and 1.2). The γ-γ links inserted by Factor XIIIa in fibrin serve as a specific antigenic marker, to which monoclonal antibodies can be generated. The usage of monoclonal antibodies imparts a high degree of specificity to the detection system, and allows the usage of whole blood, plasma, or serum as sample substrates. The original theory postulated that these monoclonal antibodies could be applied to immunologic testing systems, such as enzymatic immunoassays (EIA), for the detection thrombosis or hypercoagulable states with greater specificity and sensitivity than with previously available polyclonal antibody based testing procedures (ie FDP).[25] Early experimental work in humans with EIA designed for the quantitation of D-dimers indicated the test was beneficial in the diagnosis of several coagulation disorders including disseminated intravascular coagulation (DIC), deep venous thrombosis (DVT), pulmonary thromboembolism (PTE), and subarachnoid hemorrhage.[23,26] Generally, concentrations of D-dimers using EIA were increased in patients with these thrombogenic disorders when compared to normal controls, supporting the clinical usage of the EIA D-dimer assay in conjunction with other clinicopathologic tests and diagnostic imaging techniques. Following the rapid and widespread acceptance of D-dimer assays, several drawbacks were discovered in the original EIA detection systems. The major disadvantages identified were the length of time to obtain results (several hours),

technical training required to perform the EIA, and the economical necessity to run assays in multi-sample batches. Considering that the populations in which the D-dimer assay is indicated often consists of the critically ill and/or those requiring emergency surgery because of their underlying disease, the temporal disadvantages associated with the EIA were of sufficient magnitude to hasten the development of more rapid, point-of-care D-dimer assays.

The demand for a bedside D-dimer assay led to the evolution of latex agglutination systems, now manufactured by a wide variety of commercial laboratories and available for clinical usage. Latex agglutination assays utilize monoclonal antibodies to detect cross-linked fibrin derivatives with the D-dimer configuration.[25,29,32] Briefly, the preparation of latex agglutination test kits involves the generation of specific monoclonal antibody against cross-linked fibrin derivatives (D-dimer) by inoculating various strains of mice with purified D-dimer, and later collecting and separating the monoclonal antibody from murine ascitic fluid. This technique is described in detail elsewhere.[25,32] The monoclonal antibodies obtained are then mixed in suspension with sterile latex beads and applied to a slide with patient substrate. The presence of D-dimers in test samples causes visible agglutination of the latex beads. This methodology provides for qualitative or semi-quantitative estimates of D-dimer concentrations in the test sample by multiplying the highest dilution giving visible agglutination by the minimum detectable amount, which can be calculated from standard curves generated from EIA assays.[25] Latex agglutination assays allow for analysis of plasma, serum, or whole blood. Concentration of D-dimer has been shown to be higher when analyzed in whole blood or plasma samples compared to serum.[25-29]

The practical usage of D-dimer assays grew tremendously following validation of latex agglutination test systems in humans. Use of D-dimer as a diagnostic, therapeutic, and prognostic tool has been applied to multiple disciplines in human medicine. Many recent studies describe D-dimer testing in obstetrics/gynecology, pediatrics, oncology, infectious disease, rheumatology, nephrology/urology, gastroenterology, and

neurology.[23,36,44,46,48,53,55,57,59-61,65] However, the majority of research to date has focused on the utility of D-dimer in the identification of thrombogenic conditions or cardiovascular disorders such as PTE, DVT, DIC, ischemic stroke, and myocardial infarction.[26-43]

Several clinical studies have established the value of latex agglutination D-dimer assays as a rapid, easily performed, adjunctive test for the diagnosis of PTE, DVT, myocardial infarction, and DIC.[30-33,54] D-dimer concentrations are generally moderately to markedly elevated in patient populations with these conditions when compared to normal controls, and latex agglutination assays have been shown to rapidly identify abnormally elevated D-dimer concentrations. Correlations between D-dimer analyzed by EIA and latex agglutination assays have been shown to be highly significant in several studies.[28,31,52] Recent evidence suggests that of all the available point-of-care tests for DIC in human patients, D-dimer assays appear to be the most reliable screening tool available in patients with confirmed DIC.[57] Although reported sensitivities and specificities of D-dimer assays used in the diagnosis of the previously mentioned conditions are variable between individual methods and studies, these reports indicate that D-dimers have a negative predictive value that ranging from 95-100% in the diagnosis of PTE and DVT. Other studies refute the value of D-dimers as diagnostic tools, demonstrating that D-dimer concentrations can be normal or undetectable (using latex agglutination assays) in humans with PTE confirmed by pulmonary angiography, and that EIA D-dimer testing may not consistently detect DVT.[43,47] Discordant results between positive FDP and negative D-dimers has also been reported.[51]

D-dimer assays have also been employed as prognostic indicators in critically ill humans with a variety of diseases. Persistent, marked elevation of D-dimers in people admitted to intensive care facilities for the treatment of massive traumatic injury has been shown to correlate with the development of thrombotic complications, multiple organ failure, and death.[52,63] Another study demonstrated the ability of a serial decline in D-dimer concentration to be predictive of a clinical response to immunomodulator therapy in children with rheumatoid arthritis.[60] The degree of arteriosclerosis in patients recovering from myocardial infarction has been shown to correlate with the magnitude of D-dimer elevation in blood, but these results were later debated.[27,37]

In human neurology, blood D-dimer assays have been utilized for a variety of purposes. As DVT are a major cause of concomitant morbidity and mortality in paraplegic humans despite current widespread therapy with anticoagulants, serial D-dimer assays have been successfully performed to screen paralyzed patients at increased risk for DVT.[44] D-dimers are also used to assess relative risk of additional episodes of cerebral ischemia in patients with ischemic stroke of undetermined etiology, and to evaluate patients with asymptomatic cerebral infarction.[29,55,62] It is recently reported that increased thrombin production in the CNS may be associated with development of acute clinical dementia following cerebral infarction.[48] This study showed that markedly elevated D-dimer levels in the acute convalescent period, in conjunction with other hemostatic markers, are significantly associated with the development of infarction associated dementia.

Specific studies investigating the role of D-dimer analysis in CSF have also been performed. Identification of high levels of D-dimers in the CSF of patients with subarachnoid hemorrhage have made point-of-care testing using D-dimers valuable in the emergency evaluation of head trauma. In addition, low levels of CSF D-dimers in people with subarachnoid hemorrhage are associated with an increased incidence of delayed cerebral vasospasm following fibrinolytic drug therapy.[65] As thrombi are a major reservoir of the vasoactive substance spasmogen, fibrinolytic agents are administered to patients with subarachnoid hemorrhage to prevent cerebral vasospasm. However, increased risk of intracranial hemorrhage is associated with overly aggressive fibrinolytic therapy. Thus, monitoring of D-dimer activity in CSF may identify patient populations who would benefit from additional pharmacologic fibrinolytic therapies beyond the standard of care.[65] Measurement of CSF D-dimers in humans has also been shown to accurately differentiate traumatic lumbar puncture from other types of subarachnoid hemorrhage.[23] The ability of D-dimer assays to discern other types of pathologic CNS hemorrhage (ie secondary to trauma, inflammation, ischemia, or neoplasia) from iatrogenic blood contamination during sample procurement is still under investigation in people. If D-dimers can be demonstrated to be efficacious for this purpose, it will eliminate many diagnostic dilemmas in human and veterinary medicine.

Although the incidence of traumatic subarachnoid hemorrhage and secondary CNS parenchymal hemorrhage in veterinary species is low compared to that of humans, cases of CNS bleeding and have been reported.[67-71] Considering the mechanisms responsible for CNS hemorrhage in people also exist in veterinary species, it is likely that hemorrhagic neurologic complications will be increasingly identified with the more frequent usage of advanced imaging modalities such as computed tomography (CT) or magnetic resonance imaging (MRI).

Mechanistically, CNS hemorrhage results from compromised vascular integrity, aberrations in coagulation homeostasis, or both. Disruption of the vasculature can occur secondary to infiltrative neoplasms, inflammatory CNS disease, and spontaneous or iatrogenic trauma.[71] The distant metastatic potential of many neoplasms is potentiated by increasing ability to induce vascular endothelial disruption, and serves as a specific malignant phenotypic marker.[50] Primary and metastatic CNS neoplasia is being recognized and treated with increasing frequency in veterinary medicine, and complications arising from cancer associated CNS hemorrhage have been reported.[71] Vascular integrity may also be compromised by the ischemic damage that can result from inflammatory or neoplastic lesion induced local tissue compression.

Although pure, primary cerebrovascular disease is rare in small animal species, idiopathic intracranial thrombosis has been reported.[69] Most commonly, cerebrovascular conditions resulting in CNS hemorrhage have been secondary to metabolic diseases (hypothyroidism), congenital anomalies, thromboembolic (endocarditis), or hypertensive diseases.[68-70] Spontaneous or iatrogenic (spinal tap, epidural catheterization) trauma can also result in vascular damage and hemorrhage.

Systemic coagulation and vascular disorders are well recognized in veterinary medicine, but the clinical or occult CNS complications of these diseases are often overlooked.[71] A number of inflammatory and immune-mediated primary CNS vasculitides have been reported in the human literature, and are recognized, although rarely, in veterinary medicine.[69,75] Primary and secondary coagulopathies that have resulted in CNS hemorrhage in animals are well described.[11,24,67,71] Iatrogenically induced CNS hemorrhage from anti-coagulant therapy has also been reported.[71]

The acute and chronic pathophysiologic consequences of CNS hemorrhage can result from a variety of mechanisms. Hemorrhage significant enough to elicit a mass effect (hematoma) causes compression/displacement of vital neuronal structures, elevated intracranial pressures, and decreased blood flow. The presence of blood in CSF results in the recruitment of a variety of inflammatory mediators, and can result in fever and meningitis/myelitis/encephalitis.[71] The chronic effects of CNS hemorrhage have been the focus of much human research.[72-76] Specific hemolytic products have recently been implicated in the genesis and propagation of delayed cerebral ischemia following subarachnoid hemorrhage.[72] Hemorrhage into the CNS can also result in delayed, secondary ischemic events from induced vasospasm, and has been associated with the development of meningeal fibrosis and posthemorrhagic hydrocephalus.[65,74,76]

The definitive diagnosis of CNS hemorrhage is difficult, and requires advanced imaging modalities (CT or MRI) and often exploratory surgery.[71] The MRI characteristics of intracranial hemorrhage are well described.[71] CSF analysis may support the diagnosis of CNS hemorrhage, but as discussed earlier is subject to several pitfalls. D-dimer assays have been demonstrated to differentiate subarachnoid hemorrhage from traumatic lumbar puncture.[23] In the study by Lang and colleagues, the performance of a an LA D-dimer assay was 100% sensitive and specific in differentiating traumatic lumbar puncture from subarachnoid hemorrhage.[23] In that particular study, the LA D-dimer assay was superior to the other commonly employed laboratory tests (ie xanthochromia), and D-dimer test results correlated perfectly with computed tomographic findings.[23] In addition, the commercial LA D-dimer assay kit utilized by Lang is identical to the assay employed in the following research. In veterinary medicine, the utility of D-dimer assays as an adjunctive test in CSF analysis for the purpose of identifying CNS hemorrhage is largely unknown. As D-dimer LA assays are readily available, inexpensive, and rapid, they have potential as a screening tool for the identification of CNS hemorrhage, and deserve further study in this regard.

The treatment of CNS hemorrhage involves identification and correction of any primary pathologic process. Surgical intervention is warranted in cases of rapidly expanding hematomas that cause significant compression of neural tissue. Recent evidence suggests that following the identification of CNS hemorrhage the use of

fibrinolytic therapies may reduce the number and/or severity of chronic complications of CNS hemorrhage in humans.[36,48,65] If D-dimers prove to facilitate the identification of subdural, subarachnoid, intraparenchymal, or intraventricular hemorrhage, it may allow the institution of beneficial medical therapies prior to the performance of definitive neuroimaging procedures.

Besides conflicting results observed in some human studies, other problems with the application and performance of latex agglutination D-dimer assays have been identified since their introduction to clinical medicine. The determination of the endpoint of latex agglutination assays are highly variable based on individual experience, and thus may be subject to error when performed by inadequately trained personnel.[45] Sensitivities of latex agglutination assays may also be less when compared to EIA, but this finding has not been universal in the literature.[25,25,58] Interpretation of D-dimer assays may also be influenced by the specific pathophysiologic state of the patient. Hypoalbuminemia due to underlying disease processes such as nephrotic syndrome, sepsis, hemophilias, and immune-mediated inflammation, can effect D-dimer concentrations.[56] Medications and treatments are postulated to directly or indirectly effect D-dimer assays, but particular effects have been demonstrated in a limited number of studies.[36,42,49,51,56]

Thus, the role and intricacies of D-dimer assays in human medicine need further definition and refinement as seen from the above discussion. However, the intent of answering some questions generated in previous, current D-dimer research is being performed at an astounding rate. A literature search performed by the author using MEDLINE®, revealed 918 clinical references citing D-dimers in 1997 compared to 6,386 in 1999. The commercial diagnostic industry is also responding rapidly to the demands of the human medical field with latex agglutination and enzyme based D-dimer assays systems validated in human beings now available from more than 22 manufacturers worldwide.

The use of D-dimer assays in veterinary medicine as a diagnostic tool is still in its infancy. D-dimers are frequently mentioned in literature reviews of hematologic diseases in veterinary medicine, but current reports of D-dimer assays that have been validated for usage in clinical settings are rare. One report describes the use and validation of a latex

agglutination assay as an adjunct in the diagnosis of canine DIC, but the latex agglutination assay used in this study is no longer available.[24] A second report describes the usage of an immunoturbidometric assay for the measurement of plasma D-dimers in healthy canine subjects.[66] Recently, a private laboratory has produced a monoclonal antibody against canine cross-linked fibrin derivatives, but the product is not widely available for research use. This same laboratory has also produced a commercially available qualitative D-dimer kit for use in canine studies, which was marketed to veterinarians after this present study was completed.[a] In this study, human monoclonal antibody EIA technology[b] will be employed as the reference standard for comparison to a commercially available latex agglutination D-dimer assay[c] when used in a canine research model described following this review. According to proprietary research performed by the manufacturer of the D-dimer EIA, the test is specific and sensitive for the detection of D-dimers in canine patients.[b]

In conclusion, when considering the paucity of clinical studies in veterinary medicine addressing the effects of blood contamination on CSF analysis, the lack of agreement between published reports in the literature, and the uncontrolled study designs utilized in those reports, it is apparent that investigation into the effects of blood contamination on CSF is warranted. If the role of blood contamination on CSF analysis could be clearly defined in an experimental setting, the applications to clinical medicine would be invaluable. Validation of a latex agglutination D-dimer assay in the dog would provide additional information to clinicians and pathologists, and may have broader applications in veterinary medicine.

Chapter 2- Materials and Methods

Part 1: Normal Canine Subjects

Forty-two random source dogs were initially evaluated in this study. Animal usage was approved by the University Animal Care and Use Committee (ACUC). Prior to blood, CSF, and tissue sample collection, all dogs were maintained according to a protocol previously approved by the ACUC. All dogs were obtained for

[a] Canine D-dimer Kit, AGEN Biomedical, Ltd., Queensland, Australia
[b] DIMERTEST® Gold EIA, American Diagnostica, Inc., Greenwich CT

use in non-survival student or continuing education teaching laboratories. For the purpose of this study, all dogs were examined on the days surgical laboratories were scheduled, and all blood samples were collected after pre-anesthetics were administered. Cerebrospinal fluid samples were obtained while dogs were maintained in a surgical plane of anesthesia, and all tissue specimens were collected immediately following euthanasia by intravenous barbituate overdosage.

All dogs were determined to be clinically normal based on physical and neurologic examinations, and a minimum data base consisting of a packed cell volume (PCV), total protein (TP), blood glucose concentration (BG), and determination of blood urea nitrogen (BUN). Twenty minutes following pre-medication with acepromazine maleate[d] (0.15 mg/kg IM), glycopyrrolate[e] (0.005 mg/kg IM), and oxymorphone[f] (0.05 mg/kg IM), five milliliters (ml) of venous blood was collected from each dog by cephalic, jugular, or lateral saphenous venipuncture using a 20 gauge, 2.5 cm needle attached to a 6 ml syringe. Blood was immediately transferred following venipuncture from the syringe to the BUN reagent strip, blood glucometer sample chamber, and two microhematocrit tubes. The remaining venous blood sample was placed into sterile lithium heparin tubes.[g]

PCV was determined using the microhematocrit tube centrifugal method. Refractometry was used to measure the TP. A commercial, point-of-care glucometer[h] utilizing whole blood samples was used for determination of the blood glucose concentration. Commercial urea nitrogen whole blood reagent test strips[i] were used for the estimation of the BUN, and the protocol recommended by the manufacturer was followed. All values for the minimum data base were recorded for each study subject.

Following collection of blood samples for the minimum data base, an 18 gauge, 3.81 cm, over-the- needle teflon intravenous catheter[j] was placed in a cephalic vein of

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[c] SimpliRED® D-dimer assay, American Diagnostica, Inc., Greenwich CT
[d] Acepromazine, Abbott Laboratories, North Chicago, IL

[e] Robinul®, AH Robins Co., Richmond, VA
[f] Numorphan®, Endo Pharmaceuticals, Chadds Ford, PA

[g] Vacutainer® lithium heparin tube, Becton Dickinson and Co., Franklin Lakes, NJ
[h] Accu-Chek® Advantage, Boehringer Mannheim Co., Indianapolis IN
[i] Azostix® Whole Blood Reagent Strips, Bayer Co., Elkhart IN
[j] Angiocath™, Becton Dickinson and Co., Franklin Lakes, NJ

each dog using aseptic technique. Anesthetic induction was accomplished with either thiopental sodium[k] (average dose 16 mg/kg IV) in 32 dogs or pentobarbital[l] (average dose 25 mg/kg IV) in 10 dogs. Endotracheal intubation was performed in all dogs. Anesthesia was maintained with isoflurane[m] or by intermittent IV bolus of pentobarbital.

All CSF taps were performed using a previously described, aseptic technique using 22 gauge, 1.3 x 48 mm spinal needles.[n,1,2,5] Care was taken to insure the patency of the endotracheal tube during positioning for CSF collection. Cerebrospinal fluid was collected into sterile microcentrifuge tubes in 0.5 ml aliquots, up to a maximum of 2.5 ml from each dog. At the conclusion of all procedures scheduled for the laboratory, each dog was euthanatized by intravenous barbituate overdosage.[o] When possible (30/42 dogs), brains were harvested and fixed in neutral-buffered formalin for future histopathologic analysis within six hours of euthanasia. Unadulterated brain tissue was not obtained in all cases because some dogs were scheduled for variety of terminal neurosurgical procedures prior to euthanasia.

A 0.5 ml aliquot of CSF from each dog was examined grossly, biochemically, and microscopically within 30 minutes of collection, and all additional aliquots frozen at -80° C until further analyses were performed. Gross CSF analysis included observation of the color and transparency of all samples. A CSF glucose concentration was determined using an automated clinical biochemistry analyzer.[p] CSF total protein concentration was determined using Pyrogallol red colorimetric assay, whose methodology has been described elsewhere.[1,2,10]

The procedure utilized for the Pyrogallol red assay employed in this study was as follows. All reagents were allowed acclimate to room temperature (approximately 23° C) prior to assay. Test tubes for the reagent blank, standards, controls, and patient samples were labeled accordingly. Eighty microliters (µl) of sample was micropipetted into the appropriate tube. An equal volume of double distilled water was utilized for the reagent

[k] Sodium thiopental, Veterinary Laboratories Inc., Lenexa KS
[l] Pentobarbital sodium, Veterinary Laboratories Inc., Lenexa KS
[m] Isoflo®, Abbott Laboratories, North Chicago, IL

[n] B-D spinal needle, Becton Dickinson and Co., Franklin Lakes, NJ
[o] Euthasol™, Delmarva Laboratories, Inc., Midlothian, VA
[p] Olympus AU400®, Olympus America, Inc., Melville, NY

blank tube. Next, 2.5 ml of the reagent was placed into each tube, and then all tubes were gently mixed for 10 seconds. Reaction mixtures were allowed to stand at room temperature for 15 minutes before being placed into a spectrophotometer. Reagent blanks were placed into a clean cuvette and the spectrophotometer adjusted to read zero. Reagent blanks and patient samples were then placed into clean cuvettes, and their absorbance at 600 nm recorded and compared to a standard curve.

Cell counts on each CSF sample were performed within 30 minutes of collection using hemocytometer methodology.[2,3] Following cell counts and biochemical testing, each remaining CSF aliquot was cytocentrifuged and the pellet resuspended for cytological analysis using previously described techniques.[2] All CSF samples were dogs examined microscopically by a board-certified veterinary clinical pathologist, and differential cell counts recorded.

Part 2: Determination of CSF Test Volume

According to the manufacturer of the Pyrogallol red total protein assay system utilized in this study, as little as 10 µl of CSF can be utilized for total protein quantification. A pilot study was conducted to determine the minimum sample volume of CSF required for total protein quantification by the Pyrogallol red analytical system. CSF from two normal dogs not included in the study, due to unacceptable levels of iatrogenic blood contamination, was analyzed in a manner identical to the methodology previously described in Part 1. Following initial total protein analysis, 10 additional samples (representing volumes of 10 µl to 100 µl, in 10 µl increments) were assayed for total protein using the Pyrogallol red system. CSF controls and volume samples were assayed in duplicate for each dog. The minimum volume of CSF that was required to reliably quantify CSF total protein was determined by comparison of the experimental assays to the two dogs' CSF total protein concentration assayed by VMRCVM standard laboratory methodology. An effort was made to determine this minimum volume, as the volume of CSF required for completion of later portions of the experiment was considered to be a limiting factor, and 2.5 ml of CSF was the maximum volume of CSF collected from any study subject. The minimum volume as determined from this pilot study was utilized throughout the remaining portions of the experiment.

Part 3: Effects of Blood Contamination of CSF Parameters

Prior to each CSF dilution procedure, a 3.0 ml blood sample was drawn from a clinically normal patient for a complete blood count (CBC). Of the 3.0 ml original blood sample volume, 2.7 ml was placed into a potassium (K3) EDTA, anti-coagulant vacutainer tube,[r] and the remaining 0.3 ml was placed into a microcentrifuge tube for PCV and TP determinations. TP was determined using standard refractometry. A CBC was performed using an automated hematology analyzer.[s] This blood served as the source of RBC's used to contaminate experimental CSF specimens. The CBC, PCV, and TP results were recorded for each blood sample.

Previously analyzed CSF samples (0.5 ml aliquots) were removed from the freezer and allowed to thaw at room temperature for 15 minutes. Following thawing, 50 µl CSF samples were contaminated with serial dilutions of blood from clinically normal patients (Dog X or Y) with a known RBC count and total protein concentration to achieve final RBC concentrations of 500, 1,000, 2,000, 4,000, 8,000, 16,000, and 32,000/µl (Appendix B). Each final total sample volume used for analysis was 100 µl. The RBC concentrations chosen for analysis were deemed to represent the range of concentrations observed in clinical practice. Following addition of the various concentrations of RBC, the CSF blood mixtures were analyzed within 30 minutes.

To validate the accuracy of the RBC count using the automated hematology counter and the RBC dilution procedure, an RBC count on the first two dilutions (500 and 1,000 RBC/µl) of every sample of CSF was determined using a hemocytometer and recorded. Manual WBC counts were performed on all sample dilutions using a hemocytometer. Once CSF samples were contaminated with different RBC dilutions, protein of all sample dilutions was determined using the Pyrogallol red methodology previously described. As a negative control, identical RBC dilutions were assayed for TP using an equal volume (100 µl) of physiologic saline. This procedure was repeated in all 16 dogs whose CSF samples met the inclusion criteria for the study. Next, LA D-dimer

[r] Vacutainer® EDTA, Becton Dickinson and Co., Franklin Lakes, NJ
[s] Baker 9110, Biochem Immunosystems, Allentown, PA

assays[t] were performed on each sample dilution immediately following the TP determination, and on the blood of the dogs (X and Y) used to a source of RBC contamination. A D-dimer EIA[u] was performed on 16 random CSF samples (one dilution from each dog enrolled in the study) chosen by a technician blinded to the nature of the study, and on the blood of both dogs used to contaminate the CSF. The 16 dogs on which the EIA assay was performed on CSF also had LA and EIA D-dimer assays performed on peripheral whole blood samples.

The expected total protein concentrations obtained from this part of the experiment were then calculated using the following formula and compared to experimentally derived data to determine the effects that various RBC concentrations have on CSF total protein concentration:

$$\text{mg/dl of protein added} = \frac{[\text{serum TP (mg/dl)} \times (1.00 - \text{Hct})] \times \text{CSF RBC/}\mu\text{l}}{\text{peripheral blood RBC/}\mu\text{l}}$$

The effect of blood contamination on the CSF WBC count was also determined for all samples using the formula listed below (corrected WBC count):

$$W = WBC_f - \frac{WBC_b \times RBC_f}{RBC_b}$$

W= CSF leukocyte count before blood was added (corrected WBC count)
WBC_f = total CSF leukocyte count
WBC_b = blood leukocyte count
RBC_f = total CSF RBC count
RBC_b = RBC count blood

Following the mathematical determination of the corrected WBC count made from experimental data, a comparison between the calculated corrected WBC count and the

[t] SimpliRED® D-dimer assay, American Diagnostica, Inc., Greenwich CT
[u] DIMERTEST® Gold EIA, American Diagnostica, Inc., Greenwich CT

actual WBC count was determined for each sample in order to assess the validity of the corrected WBC formula.

LA D-dimer Assay

A D-dimer latex agglutination (LA) assay[v] was performed on each sample as described in Parts 3, 5, and 6. The test procedure for the D-dimer LA was performed in accordance with the manufacturer's recommendations.

First, all test reagents and controls were allowed to come to room temperature for at least 10 minutes prior to testing. Test samples (CSF, normal saline, peripheral whole blood) were manually mixed for 30 seconds. For each test dilution, 10 µl of sample was pipetted into one of two reaction wells on the agglutination tray marked either "negative control" or "test". Reagent dispensing bottle tips were cleansed with cheesecloth prior to each test procedure. One drop of negative control reagent was then added to the "negative control" well, and one drop of test reagent was added to the "test" well in the agglutination tray. Contents of both of the agglutination tray wells were then mixed for 5 seconds each with a plastic mixing rod, and separate rods were used for each sample well to ensure the sample mixture was evenly across the well surface. The agglutination tray was then gently rocked for 2 minutes. Each sample well was examined for the presence of agglutination and results recorded. For negative results, one drop of positive control reagent was added to the "test" well and the agglutination tray was rocked until a visible agglutination reaction was observed, or for a total of 30 seconds.

"Test" well results were considered positive if any agglutination was noted when compared to the "negative control" well. Positive reactions were further subjectively graded into weak or strong positive reactions (Figure 2.1). All positive reactions were retested and considered positive if confirmed following repeat testing. Test results from negative controls were considered invalid if agglutination was not observed following addition of the positive control reagent. This was not observed in any test sample. A test run was considered invalid if agglutination was noted at any time in the "negative control" well. Test results were interpreted as negative if no agglutination was observed

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[v] SimpliRED® D-dimer assay, American Diagnostica, Inc., Greenwich CT

when compared to the negative control, and agglutination became visible within 30 seconds following the addition of positive control reagent to the "test" well.

EIA D-dimer Protocol

EIA D-dimer assays were performed on plasma samples, saline controls, or CSF from dogs as described in Parts 3, 4, and 5. The protocol was performed in accordance with the manufacturer's recommendations.

Test wells coated with anti-D-dimer monoclonal antibody (DD-3b6) were removed from the packaging and washed with phosphate buffered saline (PBS). Next, 100 µl of PBS was added to the sample well, followed by 25 µl of D-dimer standard dilution or test sample. D-dimer sample standards were prepared according to the manufacturer's recommendations. The sample well was manually mixed for three minutes and allowed to incubate for one hour at room temperature. After completion of the first incubation period, the sample well was washed three times with PBS, with sample contents being completely emptied and blotted onto absorbent paper between washes. Tag antibody was then reconstituted, and 50 µl was added to the sample well. The well was manually mixed and allowed to incubate at room temperature for one hour. The sample well was washed again three times with PBS following this second incubation period. ATBS substrate was activated by adding 10 µl of diluted 3% hydrogen peroxide (10 µl 3% hydrogen peroxide in 100 µl distilled water) to 900 µl of ATBS substrate (for each of the 8 sample wells tested). Then, 100 µl of activated substrate was added to the sample well, and mixed for three minutes. The well was then incubated at room temperature for 20 minutes. Finally, 50 µl of stopping reagent was added to the sample well, and the absorbance was determined at 420 nm using a spectrophotometer. This procedure was repeated for each sample tested. The average absorbance value of each test sample was determined by comparison to a standard curve generated using the supplied high molecular weight D-dimer standards supplied with the test kit (Figure 2.2).

Part 4: D-dimer Concentrations in Dogs with Coagulation Disorders

Eleven dogs with suspected or documented coagulation dysfunction were the subjects of this portion of the study. This part of the study was performed to evaluate the performance of the D-dimer LA assay utilized in previous portions of the experiment (positive control). All dogs were client owned animals admitted to the VMRCVM-Veterinary Teaching Hospital for a variety of medical and/or surgical problems in which a diagnosis of a coagulopathy was made. Samples obtained for D-dimer testing were taken from extraneous blood submitted to the VMRCVM Clinical Pathology Laboratory for a number of standard clinicopathologic tests performed in clinical practice, after obtaining informed owner consent and consultation with the attending veterinarian caring for the patient.

Specific coagulopathies identified included DIC (6) and PTE (5). An antemortem diagnosis of DIC was made if the following criteria were met: presence of a clinical condition of known association with DIC, thrombocytopenia (< 120,000 platelets/µl in all cases), prolonged one-step prothrombin time (OSPT) and/or activated partial thromboplastin time (aPTT), and elevated fibrin degradation product (FDP) concentrations. Patients with PTE were diagnosed at necropsy, but were suspected to have this condition antemortem if they had a known medical condition predisposing to PTE formation, an acute onset of dyspnea and/or tachypnea, and hypoxemia (paO_2 < 60 mm Hg) on blood gas analysis. Diffuse interstitial pulmonary infiltrates were also present in thoracic radiographs obtained in 3/11 (27%) cases studied.

Specific medical conditions associated with DIC included hemangiosarcoma (HSA) in 4/6 (67%) of cases, and one case each of pancreatitis and sepsis associated with prostatic abscessation. All cases of HSA were diagnosed following histopathologic examination of tissue obtained at surgery or necropsy. The dog with pancreatitis had clinical signs (vomiting, diarrhea, pyrexia, abdominal pain), laboratory results (neutrophilic leukocytosis, elevated amylase and lipase, hyperbilirubinemia), ultrasonographic findings (enlarged, cavitated hypoechoic pancreas) consistent with a diagnosis of acute pancreatitis, and necrotizing pancreatitis was identified at necropsy. The dog with the prostatic abscess died during emergency laparotomy to repair the

ruptured abscess, and sepsis was confirmed at surgery by culturing *E.coli* from blood, spleen, liver, peritoneum, and prostatic parenchyma.

Dogs with PTE had AIHA (2/5), pituitary dependent hyperadrenocorticism [HAC] (2/5), and nephrotic syndrome (1/5). Diagnosis of AIHA was made if there was clinical evidence of hemolysis, no identifiable concurrent primary medical disorder, and at least two of the following laboratory criteria were present: spherocytosis, positive direct Coombs test, autoagglutination, and PCV < 20%. A clinical diagnosis of HAC was made if typical clinical signs (PU/PD, polyphagia, dermatologic changes) were present and ACTH stimulation testing, endogenous ACTH measurement, high-dose dexamethasone suppression testing, and abdominal ultrasonography supported the diagnosis. HAC was confirmed at necropsy in both patients. Nephrotic syndrome was diagnosed based on physical findings of ascties, evidence of hypoalbuminemia, low plasma ATIII concentration, hypercholesterolemia, and glomerular proteinuria (UP:UC = 21.3). A diagnosis of idiopathic membranoproliferative glomerulonephritis was made by percutaneous renal biopsy, and verified at necropsy.

A 3.0 ml blood sample was drawn from each dog for D-dimer analysis, and 2.7 ml were placed into potassium (K3) EDTA anti-coagulant tubes until analysis. The D-dimer LA assay was performed and interpreted on each sample in a manner identical to that described in Part 4, within three hours of sample collection.

The validity of the D-dimer LA assay system employed in this portion of the experiment was further assessed by quantifying D-dimer concentrations from this population of coagulopathy patients (and 16 healthy controls) using a D-dimer enzyme linked immunoassay system.

Part 5: Peripheral Blood and CSF D-dimer Concentrations in Dogs with CNS Disorders

Nineteen client owned dogs admitted for complaints associated with the CNS were evaluated. Samples used for analysis were taken from extraneous whole blood and CSF submitted for routine clincopathologic testing during antemortem diagnostic procedures, and informed consent was obtained from all owners.

All dogs presented with signs referable to the CNS including paraparesis/plegia (n=7), seizures (n=5), coma (n=1), central vestibular disease (n=1), hypermetria (n=1), spinal pain (n=1), and spinal fracture/subluxation (n=1). Significant physical examination abnormalities were limited to the nervous system in all dogs except dog 69 which also had a ruptured urinary bladder and comminuted left femoral fracture. Metabolic encephalopathies were excluded in dogs based on the results of a CBC, serum biochemical profile, and urinalysis. Liver function tests were also performed (bile acids or ammonia tolerance) in four cases (dogs 54, 56, 61, and 62) and were normal.

A diagnosis of granulomatous meningoencephalitis (GME) was made in 6 cases. GME was suspected in all 6 cases based on the signalment and antemortem findings of a mononuclear and/or neutrophilic pleocytosis on CSF analysis, negative serologic testing for other infectious diseases (systemic mycoses, toxoplasmosis, neosporosis, canine distemper virus), and results of intracranial imaging studies (computed tomography [CT] and/or magnetic resonance imaging [MRI]). GME was confirmed in all dogs at necropsy.

Meningiomas were suspected in three dogs based upon signalment, clinical signs, CSF analysis, and the finding of a broad based, uniformly contrast enhancing, extra-axial intracranial mass lesion on CT or MRI. Histologic diagnosis was confirmed by surgical biopsy in one case, and at necropsy in all cases.

Degenerative myelopathy (DM) was diagnosed by exclusion in three GSD who underwent clinical examination, thoracolumbar myelography and/or CT scan, and lumbar CSF analysis. Necropsy examinations were performed in all DM cases, and confirmed the diagnosis.

Intervertebral disc disease was diagnosed in four cases based on signalment, clinical signs, neurologic examination findings, results of myelography, and response to surgical decompressive therapy. A single dog with lumbar spinal fracture/luxation and progressive hemorrhagic myelomalacia (hematomyelia) was diagnosed via radiography, CSF analysis, and necropsy. A clinical diagnosis of steroid responsive meningitis (SRM) was based on signalment, history, neurologic examination findings, CSF analysis (including culture), and response to immunosuppressive corticosteroid treatment. The diagnosis of Rocky mountain spotted fever was made based on clinical findings

consistent with vasculitis and central vestibular disease, CSF analysis, acutely elevated IgM and IgG titers to *Rickettsia rickettsii*, demonstrable seroconversion on convalescent serum, and clinical improvement following treatment.

Peripheral blood (3.0 ml) was collected into K3 EDTA anticoagulant from each dog and was used to perform LA and EIA D-dimer assays. Blood D-dimer assays were performed within three hours of sample collection. CSF was also harvested from each animal in this portion of the study and LA and EIA D-dimers were performed on each CSF sample. Results were recorded for each sample.

Statistical Analysis

The CSF protein and WBC concentrations were log transformed [$\log_{10}$] to stabilize the variance. Geometric mean regression was used to estimate the relationships between red blood cell concentrations and response variables. All statistical analyses were performed using a commercial software program with the addition of the Sawada add-in for mean geometric regression, as originally reported by Riggs.[w,x,77]

Kappa values were calculated to test agreement between calculated and experimentally derived CSF WBC counts and TP concentrations; the kappa statistic was also utilized to test agreement between LA and EIA D-dimer assays in both control and diseased dogs. Table 2.1 provides guidelines for kappa statistic interpretation.[78] Table 2.2 provides formulas used in calculation of sensitivities, specificities, and positive and negative predictive values. The sensitivity is the frequency of a positive test result when the disease is present.[79] The specificity is defined as the frequency of a negative or normal test result when the disease is absent.[79] The positive predictive value (PPV) is the percentage of diseased patients with positive or abnormal test results, whereas the negative predictive value (NPV) is the percentage of disease free patients with a positive or abnormal test results.[79] The Anderson-Darling goodness-of-fit test (with 95% confidence intervals) was utilized in calculations of normal CSF reference intervals. P values < 0.05 were considered significant.

[w] SAS, SAS Institue, Cary, NC
[x] Microsoft Excel®, Microsoft Systems, Seattle, WA

Chapter 3- Results

Parts 1-3: Normal Canine Subjects, Normal CSF Reference Intervals, Determination of CSF Test Volume, Effects of Blood Contamination on CSF Parameters, D-dimer Results

Medical histories and previous uses were not available for the 42 dogs. Twenty-seven of forty-two dogs were of mixed breeding. The fifteen remaining dogs represented eight pure breeds including Beagles (n=5), Dalmations (n=2), German shepherds (n=2), Poodles (n=2), and one each of the following breeds Pit bull terrier, Jack Russell terrier, Siberian husky, and Labrador retriever. The sample population consisted of 23 males (15 intact, 8 neutered) and 19 females. Male dogs were classified as neutered if testes were not identified in the scrotum, inguinal area, or abdomen by palpation and/or visualization. Seven female dogs were spayed, as an ovariohysterectomy was performed on each of those dogs prior to enrollment in this study. Four female dogs were considered spayed based on palpation and/or visualization of a surgical scar on the ventral abdomen. Definitive reproductive status of the eight remaining female dogs was unknown.

Exact ages of dogs were unknown, but it was estimated that a range of ages (approximately 0.5 to 4 years) were represented in the sample population. Mean age of the 17 dogs was 2.4 $\pm$ 0.2 years (range, 1.5 to 4 years). Body weights ranged from 14 to 41 kg, with a mean of 24.9 $\pm$ 1.8 kg.

Only dogs with normal physical and neurologic examinations, minimum data base results, and normal CSF were included in this portion of study. Normality of CSF was determined using previously published reference ranges for CMC CSF glucose, total protein, cell counts, and morphology.[2] In addition to these criteria for inclusion, only CSF samples with WBC counts within reference ranges and RBC counts of 0-1/µl, by use of the hemocytometer, were included in an attempt to reduce interpretation complications due to blood introduced during sample procurement. Cutoff values for RBC counts considered unacceptable for this study were chosen arbitrarily. Seventeen of forty-two sampled dogs met inclusion criteria of the study, but CSF from 16/17 dogs underwent analysis because of insufficient CSF sample volume in one dog.

Fourteen dogs had minimum data base values within reference ranges for all parameters tested. Three dogs (dogs 1, 12, and 16) had PCV, TP, and BUN within

reference ranges, but had hyperglycemia. In these three dogs, a routine urinalysis was performed in each case, and was unremarkable. The degree of hyperglycemia was mild in all dogs and attributed to stress during venipuncture, and, thus, the dogs were included in the study. The mean blood glucose for the seventeen dogs was 110.4 $\pm$ 3.9 mg/dl (range, 85 to 139 mg/dl). Mean PCV was 44.1 $\pm$ 1.5% (range, 38 to 56%) and the mean TP was 6.8 $\pm$ 0.1 g/dl (range, 6 to 7.3 g/dl). All dogs had BUN concentrations < 25 mg/dl.

Based on results of a pilot study to determine CSF sample volume to be tested, 50 µl aliquots of CSF were utilized for testing throughout the experiment. The mean CSF total protein concentration of two duplicates of 50 µl CSF aliquots was 18.2 $\pm$ 0.02 mg/dl, compared with a mean of 18.3 $\pm$ 0.01 mg/dl when assayed using a standard (80 µl) sample volume as per previously validated VMRCVM clinical pathology laboratory procedure. Results using larger sample volumes were similar, and assays of sample volumes less than 50 µl failed to yield reproducible results. CSF samples considered normal on the basis of biochemical and cytologic examination were utilized to establish biochemical and cytological reference ranges for the VMRCVM clinical pathology laboratory.

CSF obtained from all dogs was clear and colorless upon visual inspection. The mean total nucleated cell count was 0.7 $\pm$ 0.2/µl (range, 0 to 2/µl). Mean RBC count was 0.3 $\pm$ 0.1/µl (range, 0 to 1/µl). The mean CSF glucose and TP concentrations were 69.7 $\pm$ 0.2 mg/dl (range, 60 to 84 mg/dl) and 11.6 $\pm$ 0.5 mg/dl (range, 9 to 15 mg/dl), respectively.

Statistically significant linear increases in CSF total protein and WBC concentrations occurred with increasing degrees of RBC contamination (Figures 3.1 and 3.2). From this data, corrected CSF total protein and WBC formulas were determined.

The relationship between CSF total protein concentration and CSF RBC concentration can be defined by the following equation:

$$\text{CSF TP (mg/dl)} = 5.70 + 0.0007\ ([\text{RBC}/\mu\text{l}])$$

Agreement between the calculated corrected TP concentration and the experimentally derived data was moderate (kappa- 0.46) but not statistically significant.

The CSF WBC concentration can be calculated using the following formula:

$$\text{CSF WBC}/\mu l = 0.45 + 0.001\ ([\text{RBC}/\mu l])$$

Agreement between the calculated corrected WBC count and the experimentally derived formula was almost perfect (kappa- 0.90) and statistically significant ($P < 0.0001$).

LA and EIA D-dimer assays performed on CSF, *in vitro* CSF-peripheral blood mixtures, and peripheral blood were negative (EIA < 250 ng/ml cutoff) for all samples tested from normal dogs.[25,66] The mean EIA D-dimer concentration in the CSF of the 16 normal dogs, prior to blood contamination, was 16.2 $\pm$ 4.3 ng/ml, with a range of 0 to 54 ng/ml, and the mean D-dimer concentration in peripheral blood was 54.6 $\pm$ 19.8 ng/ml; range, 0 to 190 ng/ml. All LA D-dimer assays were negative when performed on saline, and D-dimers could not be quantitated using the EIA when performed on saline. Complete necropsies were performed on dogs 1-11, and 17. In addition, post-mortem examinations were also performed on dogs 18-25 and 32-42, for the purposes of confirming normality in dogs utilized to establish CSF reference ranges. No significant gross or microscopic lesions were found.

Cerebrospinal fluid results from all forty-two dogs were utilized for establishment of references intervals for normal CSF. Grossly, CSF was clear and colorless in all dogs. Mean CSF total nucleated cell count (TNCC) was 1.0 $\pm$ 0.8 cells/µl. The CSF mean red blood cell count was 10.4 $\pm$ 12.6 cells/µl, and the mean CSF glucose concentration was 68.5 $\pm$ 6.2 mg/dl. CSF mean total protein concentration was 13.4 $\pm$ 3.3 mg/dl, with a range of 6 to 22 mg/dl. All 42 CSF samples were microscopically normal.

Part 4: D-dimer Concentrations in Dogs with Coagulation Disorders

This population of dogs was comprised of five spayed females, five neutered males, and one intact male with a mean age of 9.2 $\pm$ 1.0 years (range, 5 to 14 years). There were two German shepherd dogs (GSD), two Labrador retrievers, and one each of the following breeds: Golden retriever, Dachshund, Poodle, Airedale terrier, Shih tzu, Irish wolfhound, and a mixed breed.

DIC was diagnosed in 6 dogs and PTE in 5 dogs. Detectable D-dimers were present in all dogs with PTE and in 5/6 dogs with DIC, when assayed with EIA. Mean EIA D-dimer concentration was 1,093.4 $\pm$ 172.3 ng/ml (range, 0 to > 2,000 ng/ml). The

LA D-dimer assay resulted in visible agglutination in 7/11 dogs (4/5 PTE dogs; 3/6 DIC dogs). Blood EIA concentrations less than 250 ng/ml were considered normal (negative), based on results of human studies and a single study in dogs.[25,66] In cases that had quantifiable peripheral blood D-dimer concentrations, there was a significantly higher mean blood D-dimer concentration in dogs with systemic coagulation disorders (1,093.4 $\pm$ 172.3 ng/ml [range, 0 to > 2,000 ng/ml]) when compared to normal dogs (54.6 $\pm$ 19.8 ng/ml [range 0 to 190 ng/ml]), with a p value of <0.001.

As an aid in the diagnosis of DIC, the EIA D-dimer had a sensitivity of 86%, specificity of 100%, PPV of 100%, and NPV of 94%. The sensitivity, specificity, positive and negative predictive values were 100% for the EIA assay in identifying dogs with PTE. When dogs with systemic coagulation disorders were considered together, the EIA assay had an overall sensitivity of 92%, specificity of 100%, PPV of 100%, and NPV of 94%.

The LA D-dimer assay had a sensitivity of 83%, specificity of 100%, PPV of 100%, and NPV of 94% in evaluation of dogs with PTE. For dogs with DIC, the sensitivity of the LA assay was 67%, specificity was 100%, PPV was 100%, and NPV of 84%. In dogs with systemic coagulation disorders, the LA D-dimer assay had an overall sensitivity of 73%, specificity of 100%, PPV of 100%, and NPV of 80%.

The agreement between the LA and EIA D-dimer assays, when utilized in the diagnosis of coagulation disorders [PTE (kappa- 0.39), DIC (kappa- 0.21)], or all coagulation disorders (kappa- 0.29)] was fair, but not statistically significant (Table 2.1).

Part 5: Peripheral Blood and CSF D-dimer Concentrations in Dogs with CNS Disorders

The mean age of this group of 19 dogs was 8.9 $\pm$ 0.8 years (range, 2 to 15 years). There were 7 spayed females, 11 neutered males, and one intact male. The population consisted of primarily purebred dogs with the following distribution GSD (n=3), Dachshunds (n=3), Miniature poodle (n=2), Labrador retriever (n=2), mixed breeds (n=2), and one each of the following: Corgi, Maltese, Boston terrier, Siberian husky, Shih tzu, Springer spaniel, and Lhasa apso.

The EIA and LA D-dimer assays performed on peripheral blood of this group of dogs were negative, except for one dog diagnosed with RMSF (dog 72), in which both assays were positive (EIA D-dimer concentration 510 ng/ml).

For purposes of data analysis, dogs with CNS disorders were placed into three groups. Group 1 dogs (total n= 8) were considered to have infectious/inflammatory CNS diseases and consisted of dogs with GME (n= 6), SRM (n= 1), and RMSF (n= 1). Group 2 (total n= 5) was comprised of dogs with traumatic CNS injury and contained dogs with IVDD (n= 4) and the dog with a spinal fracture/luxation. Group 3 was composed of dogs with degenerative or neoplastic CNS disorders (total n= 6), and contained dogs diagnosed with DM (n= 3) or meningioma (n= 3).

In Group 1 dogs, the LA D-dimer assay was positive in 6/8 cases (both dogs with SRM and RMSF; 4/6 dogs with GME). When used as an adjunctive test for infectious or inflammatory CNS disease, the LA D-dimer assay had a sensitivity of 80%, specificity of 100%, PPV of 100%, and NPV of 88%. D-dimers were quantified in both dogs with SRM and RMSF, and 5/6 dogs with GME when using the EIA (mean D-dimer concentration 953.8 $\pm$ 204.4 ng/ml; range, 32 to > 2,000 ng/ml). The EIA D-dimer sensitivity was 88%, specificity 100%, PPV of 100%, and NPV of 94% when used to aid in the diagnosis of infectious or inflammatory CNS diseases. Agreement between the LA and EIA D-dimer assays when testing Group 1 dogs was substantial (kappa- 0.61), but not statistically significant.

The LA D-dimer assay was positive in 2/5 dogs in Group 2 (one IVDD and the spinal fracture case). When used in the diagnosis of traumatic spinal cord injury the LA D-dimer has a sensitivity of 62%, specificity of 100%, PPV of 100%, and NPV 84%. The EIA D-dimer assay was positive in 3/5 dogs with traumatic CNS injury (IVDD= 2, spinal fracture/luxation= 1). Mean EIA D-dimer concentration in Group 2 dogs was 674.4 $\pm$ 213.4 ng/ml. The sensitivity of the EIA assay in Group 2 dogs was 71%, specificity 100%, PPV 100%, and NPV 88%. There was substantial agreement between the EIA and LA D-dimer assays in Group 2 dogs (kappa- 0.62), although this value was not statistically significant.

In dogs with degenerative or neoplastic CNS disorders (Group 3), the LA D-dimer assay was negative in all cases. The sensitivity of the LA D-dimer assay in this

group of dogs was 0%, specificity 100%, PPV 100%, and NPV 72%. One dog with a meningioma had D-dimer detected in CSF with the EIA (D-dimer concentration- 844 ng/ml). The sensitivity of the EIA assay for the detection of degenerative or neoplastic CNS disease was 54%, specificity 100%, PPV 100%, and NPV 76%. Agreement between the LA and EIA D-dimer assays in Group 3 dogs was almost perfect (kappa- 0.88), but not statistically significant.

In dogs with CNS disorders, there was a trend for the mean CSF D-dimer (EIA) concentrations to be higher in Group 1, 2, and 3 dogs when compared to normal dogs, but the small sample size of the individual groups of diseased dogs precluded statistical analysis. When all dogs with CNS diseases were examined together, the mean EIA D-dimer concentration was significantly ($p = 0.03$) higher (511.6 ± 279.8 ng/ml) than normal dogs (mean 16.2 ± 4.3 ng/ml).

D-Dimer Summary Results

When all diseased dogs were analyzed together, agreement between the LA and EIA D-dimer assays was substantial (kappa- 0.61) and statistically significant ($p = 0.0003$). Differences in the quantity of D-dimers present in blood or CSF, when analyzed by EIA, were not detected in any group of diseased dogs.

Chapter 4- Discussion

Parts 1-3: Normal Canine Subjects, Normal CSF Reference Intervals, Effects of Blood Contamination on CSF Parameters, D-dimer Results in Normal Dogs

From the population of 42 dogs studied, reference ranges for normal canine CSF parameters were established, and are similar to published values.[1,2,7] Normality of dogs in this study was based on clinical and limited laboratory evaluations, and later confirmed by necropsy examination in 72% (30/42) of the dogs. Based on the results of this study, normal canine CSF should be clear and colorless on gross examination. This population of dogs had a CSF glucose concentration that ranged from 56 – 82% (mean 71%) of the blood glucose concentration, which is in close agreement with the 60 – 80% range cited in the literature.[1,2,7] Although, normal canine CSF should be free of red blood cell contamination, and was in 17/42 (41%) of dogs in this study, iatrogenic blood

contamination occurred frequently. However, the degree of blood contamination associated with collection was minimal (CSF mean red blood cell count 10.4 $\pm$ 12.6 cells/µl), and as will be discussed later, clinically inconsequential. The results indicate that CSF WBC was < 3/µl in clinically and histologically normal dogs, which is in agreement with results of a similar, clinical and necropsy based report of healthy dogs by Bailey and Higgins.[7]

Operator error and/or inexperience associated with the manual quantification of CSF cell counts could have affected the results of this study. Leukocytes tend to be larger and have irregular cellular borders when compared to erythrocytes, but microscopic differentiation between white and red cells can be difficult. RBC lysing procedures, which may alleviate this problem, were not employed in this study.[1,13,14] Results of studies in people have shown that RBC destruction occurs prior to WBC lysis in CSF samples, and delayed CSF analysis may result in RBC crenation. This sometimes leads to misclassification of an RBC as a leukocyte, resulting in an underestimation of the RBC count and overestimation of the WBC.[13] In this study, review of CSF results by a board-certified clinical pathologist, and rapid (within 30 minutes of collection) analysis of CSF were employed in an effort to reduce operator and sample preparation bias. Cell counts performed within 30 minutes of CSF collection are not subject to artifactual error associated with cellular lysis.[1-3] The addition of homologous or heterologous albumin solutions has also been advocated for the purposes of preserving CSF cellular morphology, but were not utilized in this study.[1-3]

Results indicate the reference range of CSF TP (6 to 22 mg/dl) is lower than previously reported values.[1-3,5] Elevation in CSF TP has already been demonstrated to be sensitive indicator of CNS disease, and narrowing the reference interval for normal CSF TP would increase the sensitivity of this parameter at the further expense of the specificity. [1-3,5] CSF collection and basic analysis is technically easier, economical, readily available, and more rapidly performed in clinical practice when compared to other available neurodiagnostic procedures (electrodiagnostics, contrast radiographic procedures, CT and/or MRI, tissue biopsy). Thus, the value of CSF analysis as a screening test, when combined with a complete clinical neurologic examination, for animals with suspected CNS disease is obvious. However, poor overall specificity of

CSF analysis will equate to the need for additional diagnostic procedures in the majority of cases in order to establish a definitive diagnosis.

An inherent weakness of the results obtained here is the sample size (n= 42) utilized to establish reference ranges. According to the National Committee for Clinical Laboratory Samples, 39 samples are the minimum requirement for establishment of a reference range using 95% confidence intervals, although 120 test samples is the general recommendation.[80] This population of 42 dogs was considered an adequate population to establish CSF reference ranges, as minimum sample size requirements were satisfied, clinical and biochemical normality was demonstrated in all cases, absence of morphologic disease was documented in 30/42 dogs that underwent complete necropsy examination, and numbers of dogs studied in this report exceeded those of previously published reports of normal animals.[5,7] However, when utilizing such a sample size, the major detriment is that experimentally derived data may not truly be representative of the 2.5% and 97.5% population percentile values.[80]

As hypothesized, the *in vitro* effects of varying degrees of RBC contamination on CSF TP and WBC demonstrated a statistically significant linear increase in both the CSF TP and WBC with increasing RBC concentration. The CSF TP correction formula generated from experimental data indicates that CSF TP would increase 1 mg/dl per 1,200 RBC present. This is in general agreement (kappa- 0.46) with the results of previously published studies which reported that the CSF TP can be expected to increase 1 mg/dl for every 500 – 2000 RBC present.[1-3,5] The wide range of RBC contamination reported to cause the same increase in CSF TP (standardly reported as 1 mg/dl per variable numbers of RBC, depending on the specific report) also explains the lack of perfect statistical agreement between this and previous studies.

The WBC correction formula generated in this experiment shows the addition of 1.45 WBC/ 1,000 RBC, which also is in almost perfect agreement (kappa- 0.90) with reported values. [1-3,5] The experimental correction formulas and Figures 3.1 and 3.2, however, revealed that iatrogenic blood contamination has minimal clinical impact until RBC concentrations exceed levels associated with gross evidence of hemorrhage. These results concur with previous reports which state that cell counts and standard biochemical

composition of contaminated CSF specimens are not significantly altered until RBC concentrations exceed 10,000 RBC/µl.[11,12]

It is important to mention that peripheral circulatory and hematologic status of the patient must be taken into account when applying these principles, as various physiologic and/or pathologic states such as anemia, polycythemia, hyperproteinemia, leukopenia, and leukocytosis can affect the degree of CSF TP and WBC alteration due to traumatic sample collection, and that serologic studies of CSF may be significantly adultered by introduction of peripheral blood.[1-3,14,15,20,21,81]

The results reported here also differ in that the degree of iatrogenic blood contamination was minimal (CSF mean red blood cell count 10.4 $\pm$ 12.6 cells/µl; range 0 – 46 RBC/µl) in comparison to results of previous studies.[7,11] Although results from both Bailey et al[7] and Hurtt[11] demonstrated that iatrogenic blood contamination did not occur to an extent that would alter CSF composition in the majority of cases examined, significant contamination (>1,000 RBC/µl) was sometimes seen in both normal and clinically diseased canine subjects. In the study published by Hurtt and Smith normality was based only on clinical examination, and was not supported with necropsy confirmation, as was done in this study.[11] Other possible, unexplained variables that could account for differences in degree of RBC contamination between these studies could be operator inexperience, patient anatomic confirmation (obesity, skeletal confirmation, vertebral pathology), or coexistent underlying disease (coagulopathies, vasculitis, etc.).[11]

Results from the normal dogs in the present study demonstrate that minimal iatrogenic contamination of CSF with RBC occurs (59% of samples), even when performed by experienced clinicians. The degree of RBC contamination due to collection induced trauma was of insufficient magnitude to cause significant changes in the gross character, biochemical content, or microscopic appearance of any CSF sample. When examined in conjunction with the results of the effects of *in vitro* RBC contamination of CSF on CSF TP and WBC, it can be seen that this degree of iatrogenic blood contamination is likely to be clinically inconsequential in most aspects. Although morphologic examination failed to reveal any CNS abnormalities in the 42 dogs of this study, specific quantification of CSF proteins or functional studies evaluating the

integrity of the BBB (IgG index, AQ) were not performed in this study. Thus, the potential impact of a minimal degree of peripheral blood contamination on serologic or molecular biologic CSF tests could not be fully discounted, especially when considering the extreme sensitivity and specificity of certain genetic tests.[81]

As demonstrated by this and other studies, minimal to moderate peripheral blood contamination does not significantly alter CSF composition. An important clinical implication of this finding is that elevated CSF WBC counts or protein concentrations are sensitive markers of CNS disease, are not artifactually altered by moderate amounts of blood contamination. However, as advanced ancillary CSF molecular and genetic tests are validated and become more widely available, the effect of iatrogenic blood contamination will require further investigation.

Previous reports in the human literature state that D-dimers are not demonstrable in CSF by any methodology in cases of acute, iatrogenic blood contamination, and these results were confirmed by the present study.[23] This finding supports the clinical use of rapid LA D-dimer assays for further differentiation of pathologic CNS hemorrhage from traumatic CSF collection. The identification of a more sensitive and specific method for the diagnosis of pathologic CNS hemorrhage is highly desirable, as the diagnostic limitations of finding xanthochromia and erythrophagocytosis in CSF specimens have already been discussed.

Although previous veterinary reports of hemorrhagic CNS complications have consisted primarily of single case studies, the evolution of more sophisticated and readily available neuroimaging procedures has resulted in an increased awareness of the possibility of CNS hemorrhage (subdural/subarachnoid hematomas) requiring aggressive medical or surgical therapy.[70,71,82] Hemorrhagic and thrombotic CNS complications may result in compromised neurologic function by a direct mass effect, promoting and exacerbating inflammation, reducing or inhibiting perfusion, and initiating secondary disease cascades (ie delayed vasospasm, fibrosis). Rapid diagnosis and treatment of these disorders has resulted in improved clinical outcomes in humans with a variety of underlying disorders, and may be applicable to veterinary species as well.[71-76] With the potential to rapidly identify pathologic CNS hemorrhage, performance of a screening D-dimer CSF assay could serve three primary clinical functions: raise clinical awareness

for the need for more definitive neuroimaging procedures to classify and localize the underlying pathology, initiate appropriate early treatment when indicated, and possibly prevent unnecessary diagnostic imaging in patients with negative D-dimer assays. The technical, practical, and theoretical limitations of the D-dimers assays and their relevance to this study will be discussed in the following section.

Parts 4-5: D-dimer Results in Dogs with Systemic Coagulation Disorders and CNS Diseases

Results of this portion of the study indicate that usage of D-dimers to facilitate the diagnosis of DIC and PTE in dogs is both sensitive and specific, a finding that is supported by the human literature.[26,27,31-33,54] The PPV of the EIA and LA D-dimer assays were 100% in all cases tested, although the EIA D-dimer assay consistently had a superior NPV when compared to the D-dimer LA. A preliminary investigation of the utility of an immunoturbidometric D-dimer assay for the diagnosis of DIC in dogs reported an 82% sensitivity, a value similar to the one obtained for the EIA used in the present study (86%).[24] The high overall sensitivity (92%) and specificity (100%) of the EIA D-dimer assay when used to identify dogs with systemic coagulation abnormalities in this study may have valuable clinical applications as a screening test for conditions such as DIC or PTE.

Several possibilities exist that could explain discordance between LA or EIA D-dimer assays in patients with systemic coagulation disorders. Hypoalbuminemia, defined as an albumin concentration of < 2.3 g/dl for the purposes of this study, is a common biochemical finding in both humans and dogs with severe illness.[49-51,54,56] Increased fibrinogen, lipoproteins, factor VII, blood viscosity, and arachadonic acid activity, as well as decreased antioxidant and heparin-like activity are changes that have been directly and indirectly associated with hypoalbuminemia in humans, resulting in increased platelet aggregation and endothelial injury, both of which have procoagulant effects.[56] Thus, hypoalbuminemic conditions are a well known predisposing factor to the development of hypercoaguable states in human and veterinary medicine, and can result in increased D-dimer concentrations. However, D-dimer concentrations were negatively correlated with

the albumin concentration in humans with PTE associated with nephrotic syndrome and certain vasculitides.[56,83] The precise mechanism by which D-dimer concentrations are altered by hypoalbuminemia is currently unknown.

When considering the possible effects of serum albumin concentration on D-dimers, it is interesting that in four dogs in the present study with systemic coagulopathies (nephrotic syndrome and PTE; splenic hemangiosarcoma, pancreatitis, and sepsis associated with DIC) that had negative LA D-dimer assays, each had clinically significant degrees of hypoalbuminemia (mean 1.8 $\pm$ 0.32 g/dl).

The presence of hyperbilirubinemia is an additional biochemical abnormality that has been reported by manufacturers to negatively interfere with determination of D-dimers by EIA by spectrophotometry.[a] In the present study, the EIA D-dimer assay was negative in one dog with a confirmed systemic coagulopathy (DIC). This animal was clinically icteric and diagnosed with pancreatitis associated with secondary extrahepatic biliary obstruction, which may have resulted in failure to detect D-dimers with the EIA (false negative result). However, this case also had a negative LA D-dimer, an assay which is reportedly not subject to error in the presence of hyperbilirubnemia. Thus, the possibility this test may have been truly negative must be entertained. In addition, two dogs with AIHA and PTE also had clinical and biochemical evidence of hyperbilirubinemia, but in these animals D-dimer assays performed by both methodologies were positive. Thus, the effects of hyperbilirubinemia on EIA D-dimer assay are ambiguous based on results of this study.

False positive LA D-dimer test results could have possibly been obtained in one of the dogs with AIHA, as this patient was noted to have clinical evidence of autoagglutination. As interpretation of the LA D-dimer assay revolves around subjective observation of agglutination, an autoagglutinating sample could be erroneously evaluated as positive. The probability of the result actually being a false positive in this dog is reduced when the elevated levels of D-dimer quantified by EIA, which was performed on plasma and therefore was not subject to error associated with autoagglutination, and the extreme specificity of the LA D-dimer.

The LA D-dimer is sensitive and specific for the diagnosis of DIC in the human literature, although its sensitivity is consistently lower than EIA based assays, which is an

additional reason for discordant results.[51] One particular human study reported that discordant results (elevated FDP and negative LA D-dimer assays) occurred in 25% of 763 of humans evaluated with DIC.[51] That report utilized similar criteria (in addition to others) as the ones employed in this study to establish an antemortem diagnosis of DIC, and cited accelerated fibrinogenolysis without secondary fibrinolysis and elevated fibrin monomer concentrations as potential reasons for discordance.[51] The presence of certain circulating substances, in particular rheumatoid factor, may also confound D-dimer interpretation.[51,60] The complicating conditions cited in those studies do occur in animals, procedures to evaluate them as possible reasons for discordance were not performed in this study, so their prevalence in the experimental population and possible effects on D-dimer analysis are unknown.

Technical pitfalls have been cited as contributors to discordant results in D-dimer analysis. Although the manufacturers of many D-dimer assays claim equal accuracy of their assays when performed on whole blood, plasma, or serum, studies have suggested that the usage of different sample substrates will produce significantly different results depending on the particular assay system utilized and the underlying clinical condition.[26,28,35,37,64] One particular substrate or assay has not as yet been identified as clearly superior to others, but controlled studies critically evaluating these parameters have not been performed. One human study also cited that operator inexperience contributed significantly to a decrease in the sensitivity of LA D-dimer assays.[45] Thus, test substrate selection and operator dependency errors could have played a significant role in test result discordance in this study.

In addition to the adverse influences of the aforementioned pathophysiologic alterations on D-dimer analysis, various medical therapies have been cited as altering the diagnostic utility of both LA and EIA D-dimer assays. Corticosteroid therapy is implicated as a risk factor for the development of thrombotic conditions in veterinary and human medicine. Chronic steroid usage has been associated with abnormal coagulation and fibrinolysis in humans with inflammatory bowel disease.[53] Synthetic and natural colloid administration have been reported to adversely effect D-dimer analysis,[42,49] although an additional study found no significant change in laboratory assessment of coagulation and fibrinolysis in patients receiving human albumin infusions.[56] Four dogs

evaluated in this study ultimately diagnosed with PTE and positive antemortem D-dimer assays (LA and EIA) were either receiving immunosuppressive dosages of corticosteroids for the treatment of their underlying disease (n=2, with AIHA) or were subjected to excessive endogenous steroids (n=2, with HAC). The exact role of exogenous and/or endogenous steroid influence on D-dimer analysis could not be determined due to the low number of dogs evaluated in this study, but merits further research efforts.

Multiple studies describing instances of negative LA D-dimer assays in the face of proven, significant systemic coagulation dysfunction exist in the human literature.[26-27,43-45,47,50,51] Normal human beings typically have circulating D-dimer concentrations less than or equal to 250 ng/ml, and therefore, commercial LA D-dimer assays are, in general, manufactured with the intent to detect D-dimer concentrations > 250 ng/dl.[25] Several studies have questioned the accuracy of LA D-dimer assays in people with clinically documented coagulopathies and concentrations of D-dimer only slightly above normal when quantified using various immunoassay systems.[25,27-33]

Results of this study indicate that this does not appear to be a problem with the D-dimer assay kit utilized, as cases with discordant results (ie positive EIA and negative LA D-dimer) occurred in animals with high blood concentrations (>850 ng/ml) of D-dimer. The one case of DIC in which EIA quantification demonstrated an only slightly abnormal D- dimer concentration (420 ng/ml) was positive when analyzed by LA D-dimer. Interpretation of these results must be cautious because the reference range considered normal in this study for D-dimer concentrations was extrapolated from human data, as a normal reference range for D-dimer concentrations was not validated using the recommended minimum sample number for the particular purposes of this study. Recently, investigators validated an immunoturbidometric D-dimer assay for usage in dogs, and reported that D-dimer concentration in normal dogs ranged from 20 – 280 ng/ml. These values very similar to normal humans, and the reference range considered normal for this study (mean 54.6 $\pm$ 19.8 ng/ml; range, 0 to 190 ng/ml).[66] However, an assay system that has not currently been validated for use in dogs was utilized in this study.

In this study, a fair, but not statistically significant, overall agreement (kappa-0.29) between LA and EIA D-dimers in the diagnosis of dogs with systemic coagulation

abnormalities was observed. This can be explained by a variety of inherent pathological processes, assay system imprecision, technical difficulties, and/or concurrent medical treatments. The high sensitivities and specificities of LA and EIA D-dimer assay systems previously reported must also be interpreted with caution because of a major inherent weakness of the study, small sample size. Prospective, blinded trials designed with the intent of statistically evaluating various D-dimer assays would be difficult because of problems associated with the accurate antemortem identification of DIC and PTE with currently available laboratory tests and diagnostic imaging modalities. In a 2.5 year period, only 11 cases of confirmed DIC and PTE were prospectively identified by the author in a case biased, tertiary referral care facility amongst the approximately 21,000 small animal admissions that occurred during the same years. In a retrospective analysis by the author of small animal admissions over the same 2.5 year period, the prevalence of DIC was determined to be 1.9% (396/20865 cases), while the prevalence of PTE was 0.8% (166/20865 cases). These determinations likely underestimate the true prevalence of DIC and PTE, as they reflect only records in which the diagnosis was specifically coded.

The true incidence of PTE and DIC is likely vastly underestimated in small animal species due to subclinical and/or chronic presentations, and the relatively insensitive methodologies currently available for diagnosis.[66] This finding obviously defines the potential role for D-dimer analysis in clinical veterinary medicine. In humans, D-dimer analysis has been shown, when used in conjunction with clinical physical examination, complimentary laboratory assessments, and a variety of diagnostic imaging procedures to be extremely valuable in the diagnosis of a variety of clinical and subclincal disorders including DVT, PTE, DIC, ischemic stroke, and myocardial infarction in at risk patients.[28-45] Additionally, abnormal D-dimer concentrations have been shown to an independent risk factor for severe morbidity or mortality in patients with infectious bacterial disease, obstetric complications, neoplasia, traumatic injury, and various types of shock.[46,50,52,57,59,61,63] D-dimers also show promise in the early identification of patients with impending coagulation dysfunction, and in the evaluation of therapeutic responses to several immune-mediated diseases.[53,54,60] The potential for D-dimer analysis as an aid in the early and rapid identification of systemic coagulation

dysfunction in veterinary medicine is enormous, but future investigations enrolling large numbers of patients will be required to critically evaluate their place in the clinical veterinarian's diagnostic armementarium.

In this study, both the LA D-dimer and EIA assays were 100% specific and had a 100% PPV when used to identify dogs with inflammatory/infectious, traumatic, or degenerative/neoplastic diseases. In addition, when all dogs with CNS disorders were analyzed together, the mean EIA CSF D-dimer concentration was significantly ($p = 0.03$) higher (511.6 $\pm$ 279.8 ng/ml) than normal dogs (mean 16.2 $\pm$ 4.3 ng/ml), which supports the role of intrathecal finbrinolysis in the pathophysiology of a variety of neurologic diseases. The extremely variable sensitivities (0 - 88%) of LA and EIA D-dimer tests may limit the usefulness of D-dimer analysis in the evaluation of dogs with CNS disorders, but also can be partially explained by the primary disease process occurring in individual patients.

Considering the current level of knowledge regarding the pathogenesis of CNS infectious and inflammatory disorders, it is not surprising that the dogs with RMSF, SRM, and GME had positive CSF D-dimers. When used as an adjunctive test for infectious or inflammatory CNS disease, the LA D-dimer assay had a sensitivity of 80%, specificity of 100%, PPV of 100%, and NPV of 88%. D-dimers were quantified in both dogs with SRM and RMSF, and 5/6 dogs with GME when using the EIA (mean D-dimer concentration 953.8 $\pm$ 204.4 ng/ml; range, 32 to > 2,000 ng/ml). The EIA D-dimer sensitivity was 88%, sensitivity 100%, PPV of 100%, and NPV of 94% when used to aid in the diagnosis of infectious or inflammatory CNS diseases. Intrathecal immunoglobulin production, vascular endothelial damage, and the activation of lymphocytes with resulting elaboration of pro-inflammatory cytokines are known to occur in dogs with SRM.[84] Native CNS tissues, such as microglia and astrocytes are also capable of the production and release of pro-inflammatory cytokines (TNF-α, IL-6) in response to CNS trauma, infection, or immune-mediated inflammation.[85] Any or all of these pathologic alterations could have resulted in the activation of fibrinolytic system and generation of D-dimers. In the animal with RMSF, similar mechanisms could have played a role in CNS D-dimer formation.[85,86] In addition, the RMSF agent, *Rickettsia rickettsii,* has been shown to be able to induce coagulation and resulting fibrinolysis by induction of

endothelial cell tissue factor expression.[87] However, this dog also had elevated peripheral concentrations of D-dimers and supportive clinical and laboratory evidence of DIC. Therefore, it is unknown if the CNS D-dimers were truly representative of intrathecal fibrinolysis, a reflection of increased blood brain barrier permeability, or the result of peripheral blood contamination of the CSF during sample procurement.[85]

The majority of dogs with GME also had elevated D-dimer concentrations when evaluated by the LA assay (4/6) and EIA assays (5/6). Some degree of activation of the fibronolytic system is expected in dogs with GME considering the inflammatory infiltrate that is the histologic hallmark of the disease, and its anatomic association with the vasculature of the brain, meninges, and spinal cord.[85] The elaboration of pro-inflammatory cytokines by the granulomatous infiltrate or reactive CNS supportive tissues may result in activation of the coagulation or fibrinolytic systems, causing an increase concentration of intrathecal D-dimers.[85]

The potential technical and analytical errors previously described in the section on coagulation disorders also applies to the D-dimer analysis of CNS disorders. As in some of the animals with corticosteroid responsive systemic diseases, 3/6 of the dogs with GME selected for study had received glucocorticoid therapy prior to D-dimer analysis, which could have resulted in a false-positive D-dimer result.

When used to evaluate dogs with traumatic spinal cord injury (Group 2), the LA D-dimer had a sensitivity of 62%, specificity of 100%, PPV of 100%, and NPV 84%, and was positive in 2/5 dogs tested. The EIA D-dimer assay was positive in 3/5 dogs with traumatic CNS injury (IVDD= 2, spinal fracture/luxation= 1). As expected, both D-dimer assays were positive in the dog with hematomyelia and the case with traumatic subarachnoid hemorrhage. It is interesting to note that 2/5 animals with traumatic CNS injuries (both had IVDD) had normal concentrations of CNS D-dimer when evaluated with the EIA assay, or had negative D-dimer assays. Internal (ie intervertebral disc protrusion) and external (spinal fracture/luxation) traumatic events are known to cause CNS inflammation and BBB breakdown. CNS trauma can contribute to the generation of inflammation and thus activation of the coagulation and fibrinolytic systems by both direct and indirect mechanisms. The inciting primary injury can cause direct damage to neurons, supportive elements, and vasculature.[84-85] This event can initiate a cascading

series of self-perpetuating reactions that culminate in a loss of local environmental blood flow autoregulation, activation of the arachadonic acid cascade, lipid peroxidation, aberrations in excitatory and inhibitory neurotransmitters, abnormal intracellular calcium homeostasis, and finally, neuronal, glial, and endothelial cell death.[85] As all of the dogs in Group 2 were treated with corticosteroids prior to admission to the hospital and CSF sample procurement, it is possible that false positive D-dimer results could have been obtained on that basis.

In accordance with our hypothesis, none of the dogs with degenerative myelopathy had abnormally elevated concentrations of D-dimer in CSF. Considering what is known about the pathophysiology of DM, it is not surprising that D-dimers were not elevated in these patients, as disordered coagulation and fibrinolysis are not a recognized feature of the disease.[89,90] DM was elected for study as a degenerative process mainly because of the relatively high frequency of diagnosis in clinical patients compared to other neurodegenerative disorders. In addition, some veterinary neurologists currently advocate treatment of DM with anti-fibrinolytic drugs (ie aminocaproic acid), and anecdotally report marginal to marked success with their usage.[90,y] Thus, it was also because of this potential association that DM was studied. None of the dogs with DM in this study had received any medical therapy prior to blood or CSF sampling.

It was interesting that elevated D-dimers were documented in only one dog with intracranial neoplasia when assessed by EIA. Direct tumor-induced vascular compression, elaboration of pro-inflammatory mediators, production of metastatic regulators such as matrix metalloproteinases or pro-angiogenic agents (ie vascular endothelial growth factor) by neoplastic cells are all potential initiators of coagulation and fibrinolysis.[75,85] Tumor-related subarachnoid hemorrhage is a known complication of space-occupying meningeal neoplasms in humans, likely related to tumor induced meningeal inflammation.[65,67,74,76] Two of the dogs with meningiomas examined in this study had antemortem computed tomographic evidence of CNS hemorrhage associated with their tumors, and ultimately had necropsy evidence of hemorrhagic tumor necrosis on necropsy, but had negative LA D-dimer and EIA assays. It is currently not known why D-dimers were not detected in the CSF of these patients.

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[y] Neurology IChat Server, ©1997 University of Florida College of Veterinary Medicine, Gainsville, FL

Several possible mechanisms exist to explain false negative CSF D-dimer results. The interpretation of the LA D-dimer assay is based upon documentation of visible agglutination of blood in the sample. Thus, low RBC counts observed in many of the CSF specimens may have precluded visible macroagglutination. However, the precision of the EIA procedure, should have detected elevated concentrations of D-dimers in those CSF samples with low numbers of RBC's, a phenomenon which did occur in several cases. Thus, the degree of CNS hemorrhage or blood contamination of the CSF sample may adversely affect the LA D-dimer assay. Secondly, a major assumption in the interpretation of CSF is that the disease process must be present in, or continuous with the subarachnoid space, or the BBB must be disrupted in order to produce pathologic changes that will be reflected in CSF.[1-3] It is possible that CNS pathology can be anatomically sequestered from CSF, although this phenomenon is uncommon in veterinary medicine considering the sensitivity of CSF analysis in the detection of CNS disease.[1,6,8] This may explain the negative results in one of the dogs with GME, which had obstructive hydrocephalus documented at necropsy examination, although this dog's CSF analysis was consistent with GME.

A limitation in this study was the minimal recommended number of dogs used to define the normal reference range for CSF D-dimers in this study. Intrathecal D-dimer concentrations are similar to those in blood in humans.[23] Considering that the CSF D-dimer concentration was significantly higher in all dogs with CNS diseases when compared to normal controls in this study, a difference may exist between species in their intrathecal D-dimer concentrations. Our results suggest that intrathecal D-dimer concentrations (0 to 54 ng/ml) are lower than peripheral blood (0 to 190 ng/ml) D-dimer concentrations in normal dogs. Considering these results, the sensitivity the LA D-dimer assay would need to be increased in order to avoid false-negative results. Defining abnormal D-dimer concentrations as > 250 ng/ml in this study may have resulted in a significant number of false-negative CSF D-dimer results in dogs with CNS diseases.

As was the case with D-dimer analysis in dogs with systemic coagulation disorders, the low numbers of animals with CNS diseases studied precluded meaningful statistical analysis of the data between individual groups. However, the results suggest that CNS fibrinolysis occurs in animals with inflammatory, neoplastic, and traumatic

diseases in the absence of systemic fibrinolysis. Considering the movement in human medicine to rapidly identify and treat secondary effects of CNS disease, D-dimers may have a place in the future of veterinary clinical neurology. However, additional studies are required before the utility of D-dimers as a veterinary diagnostic tool can be defined. The recent development of a canine monoclonal antibody based D-dimer assay, although not currently validated, may allow more specific application of such tests.

Chapter 5- Future Directions

As can be seen from the results of this study, and from the relatively few published studies that currently exist in the veterinary literature, further validation and rigorous assessment of D-dimer assays are required before their clinical utility can be defined. The results of an enormous body of research human medicine regarding D-dimers continually provide novel indications for the assays, while simultaneously alerting the clinician to their previously unrecognized limitations.[23-54]

With the advent of a commercially available canine monoclonal D-dimer antibody, rapid, easily performed point-of-care D-dimer determinations are widely available to practicing veterinarians.[z] Preliminary reports concerning this canine D-dimer assay from several central based laboratories, including the clinical pathology laboratory at the VMRCVM, are encouraging. However, no published reports on the validation, sensitivity, or specificity of the canine D-dimer assay exist in the veterinary literature. In an effort to further assess the performance of this new canine D-dimer assay, clinical patients undergoing coagulation testing at VMRCVM have had D-dimer assays concurrently performed, the results recorded, and aliquots of plasma banked from all prospectively identified patients. Because the D-dimer domain assayed using EIA technology is homologous amongst the species examined to date, it is hypothesized that D-dimer concentrations from patients can be quantified using the human monoclonal based antibody kit utilized in the previous research as a method a validating the canine antibody test.[34]

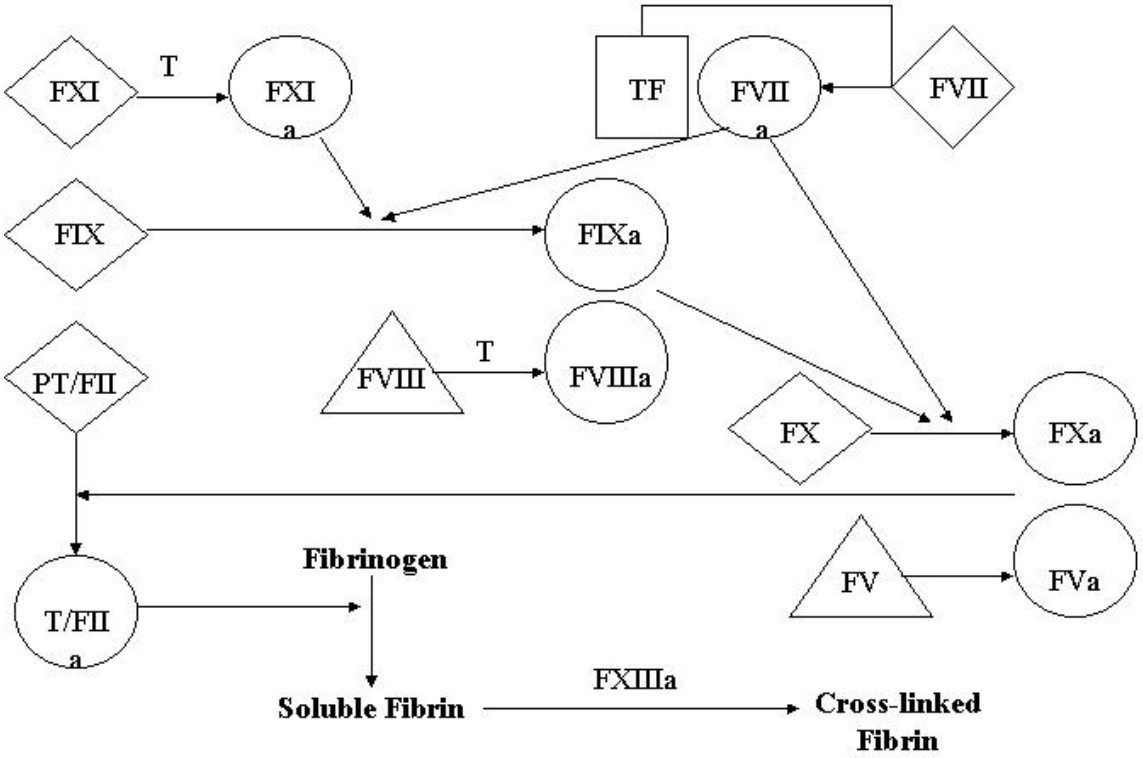
The role of D-dimer analysis in the diagnosis of canine central nervous system diseases could be further examined by quantitatively assaying D-dimer using human

[z] Canine D-dimer Kit, AGEN Biomedical, Ltd., Queensland, Australia

monoclonal EIA in stored CSF samples from client-owned patients evaluated by the clinical neurology service. The relationships, if any, between D-dimer concentrations and specific CNS diseases or CSF cytologic findings, as determined from the animal's medical record, can be further characterized. As CSF analysis is a routine part of the evaluation of most clinical patients with CNS disease, sample numbers should be adequate for statistical analysis.

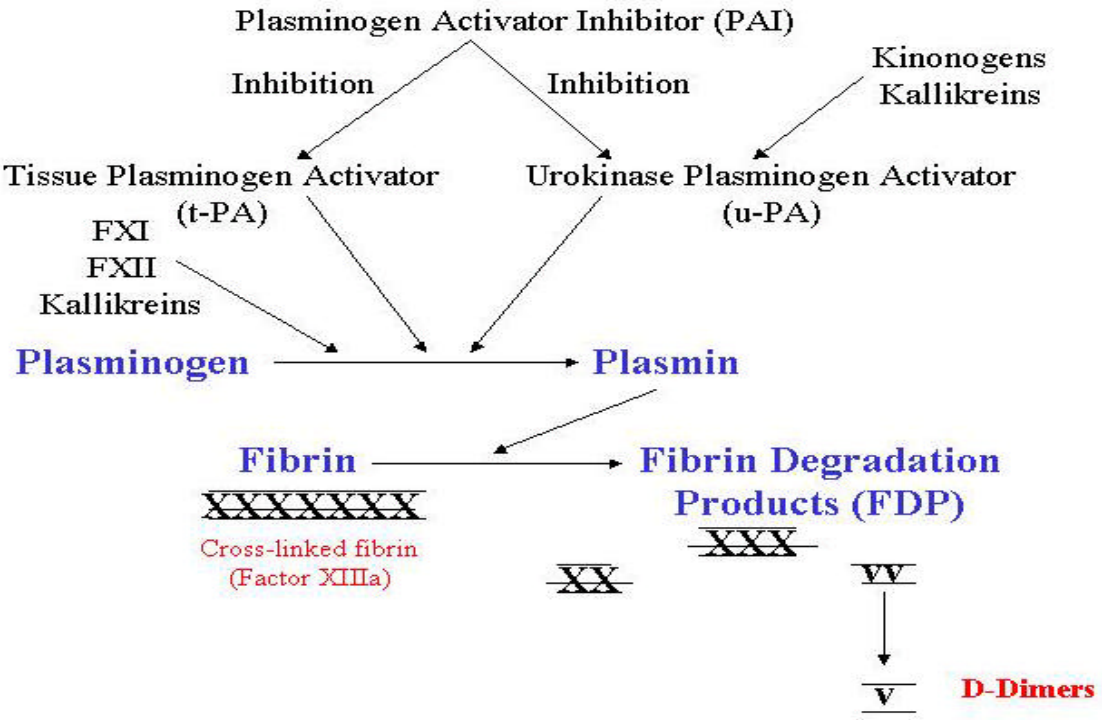
An additional opportunity exists to prospectively study and compare the efficacies of human and canine point-of-care based D-dimer assay kits in a clinical setting. Both the LA D-dimer assay utilized in this study and the canine D-dimer test now commercially available are technically easy to perform and yield rapid results. It is proposed that these assays can be simultaneous performed in all canine patients admitted to the VMRCVM intensive care ward with clinical, diagnostic imaging, or laboratory evidence of coagulation dysfunction or thromboembolic disease, or in patients receiving anti-coagulant therapies in which the attending clinician has ordered further laboratory assessment of coagulation. As EIA based assays and anti-factor Xa assays are considered the gold standards for the measurement of D-dimer concentrations and the efficacy of anti-coagulant therapies, respectively, plasma and CSF from these additional clinicopathologic tests can be saved and assayed for D-dimer and anti-factor Xa concentrations at a later date using EIA, and the results of all assays compared.[45,55,63,91]

Figure 1.1- Coagulation cascade.



TF = Tissue Factor
a = Denotes activated coagulation factor
F = Prefix indicating specific coagulation factor indicated in Roman numerals
PT = Prothrombin
T= Thrombin
Circles indicative of activated factor and/or cofactor
Triangles indicative of inactive cofactor
Diamonds represent inactive factor

Figure 1.2- Fibrinolysis and Generation of D-dimers.



F = Prefix indicating specific coagulation factor indicated in Roman numerals

Figure 2.1- Objective visual interpretation guide for LA D-dimer assay.

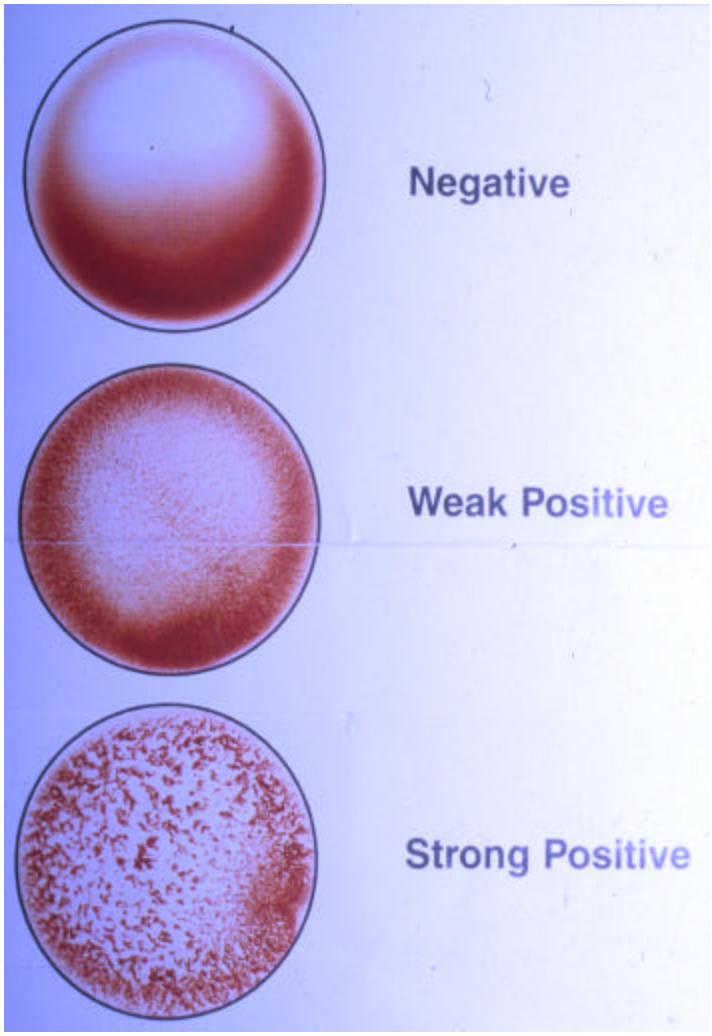
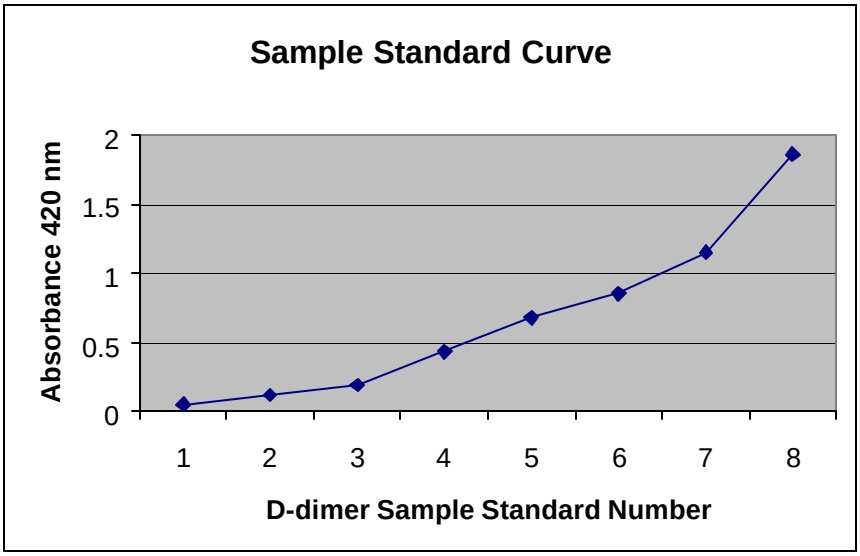
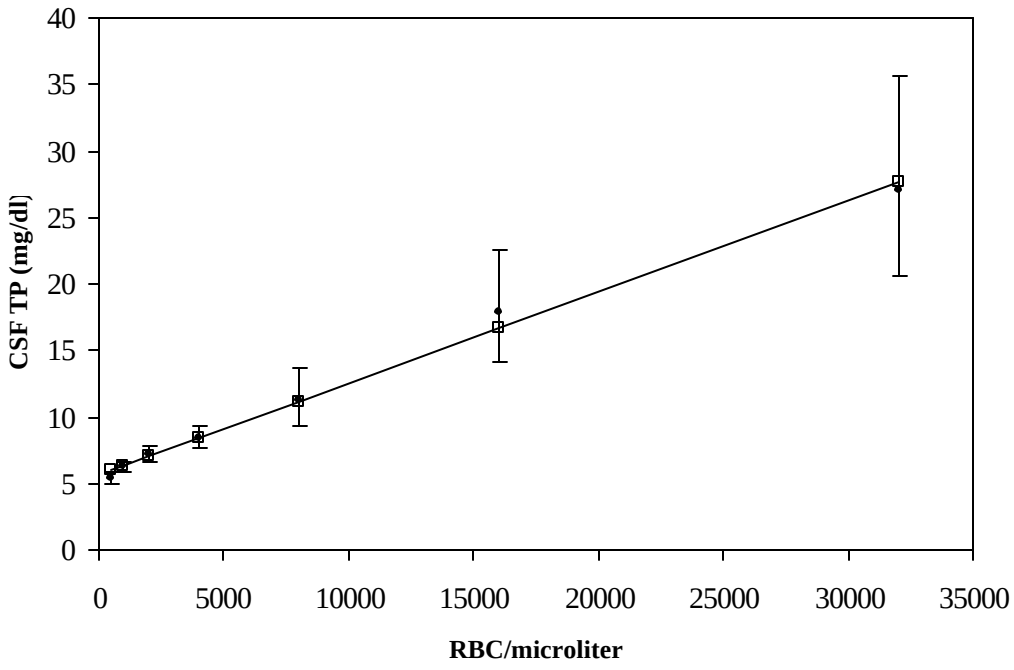


Figure 2.2- Experimental standard curve for EIA D-dimer assay.



Sample Number	D-Dimer Standard (ng/ml)	Absorbance at 420 nm
1	0	0.05
2	32	0.12
3	63	0.19
4	125	0.43
5	250	0.68
6	500	0.85
7	1000	1.15
8	2000	1.86

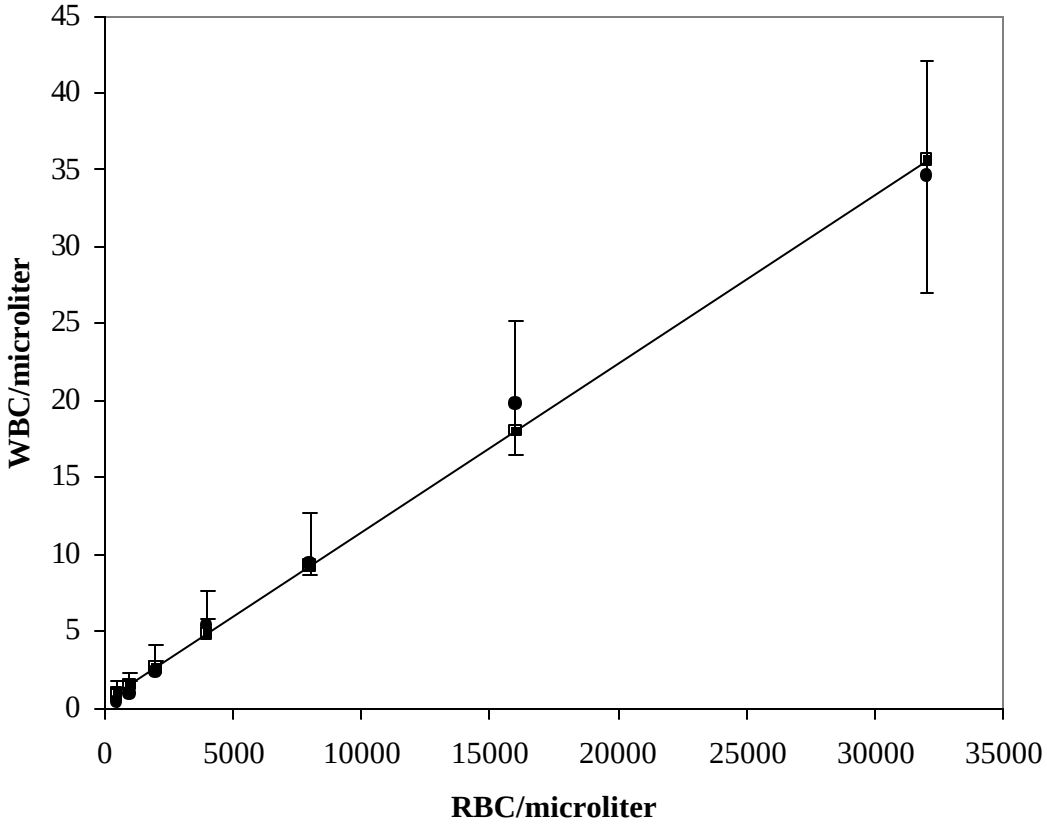
Figure 3.1- Graphic representation of statistically significant (p = 0.0003) linear relationship between increasing degrees of *in vitro* RBC contamination on CSF total protein concentration.



CSF TP = Cerebrospinal fluid total protein concentration

RBC = Red blood cell count

Figure 3.2- Graphic representation of statistically significant (p = 0.001) linear relationship between increasing degrees of *in vitro* RBC contamination on CSF total WBC count.



WBC = White blood cell count

RBC = Red blood cell count

Table 2.1- Kappa statistic interpretation.[78]

Kappa Value	Strength of Agreement
0	No better than chance
0.01-0.20	Slight
0.21-0.40	Fair
0.41-0.60	Moderate
0.61-0.80	Substantial
0.81-0.99	Almost perfect
1.00	Perfect

Table 2.2- Calculations of sensitivity, specificity, PPV, and NPV.[79]

Test Parameter	Definition	Abbreviation Key
Sensitivity	[TP / (TP + FN)] x 100	TP= True positive
Specificity	[TN / (TN + FP)] x 100	TN= True negative
Pos predictive value (PPV)	[TP / (TP + FP)] x 100	FP= False positive
Neg predictive value (NPV)	[TN / (TN + FN)] x 100	FN= False negative

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Appendix A- Biochemical composition of canine and feline cerebrospinal fluid.[1]

Species	pH	Ca	P	Mg	Cl	K	Glucose	Urea	Protein
Canine	7.42	6.56	3.09	3.00	105	2.98	74	3.56	<25
Feline	7.51	5.2	3.42	1.33	109	2.98	85	6.76	<20

Ca = calcium in mg/dl

P = inorganic phosphorus in mg/dl

Mg = magnesium in mg/dl

Cl = chloride in mEq/L

K = potassium in mEq/L

Appendix B- Blood contamination dilutional procedure.

Donor X [RBC]*	Dilution Volume#	[RBC] Dilution	Initial CSF Volume	Volume Dilution Added to CSF	Final Test [RBC]	Final Test Volume
6.31 $x10^6$	6,310	1,000	50	50	500	100
6.31 $x10^6$	3,155	2,000	50	50	1,000	100
6.31 $x10^6$	1,578	4,000	50	50	2,000	100
6.31 $x10^6$	789	8,000	50	50	4,000	100
6.31 $x10^6$	395	16,000	50	50	8,000	100
6.31 $x10^6$	198	32,000	50	50	16,000	100
6.31 $x10^6$	99	64,000	50	50	32,000	100

[RBC] = Concentration of red blood cells/µl

Diluent = 0.9% NaCl (ml)

Volumes reported in µl unless otherwise noted

#Volume reported in ml

CSF = Cerebrospinal fluid

*The dilution procedure was repeated in an identical manner for CSF samples tested using blood from Donor Y.

Vita

John Rossmeisl was born on June 25, 1971 in Springfield, Massachusetts, and has resided in several New England States including Massachusetts, New Hampshire, and Maine. He received his Bachelor of Arts degree from the University of New Hampshire in 1993, with plans to pursue graduate studies in veterinary medicine. John next attended the Auburn University College of Veterinary Medicine, from which he received the degree Doctor of Veterinary Medicine with honors in 1997. In 1998, he partcipated in a rotating small animal internship at Purdue University, and then moved to Blacksburg, Virginia where he now resides. During his tenure at Virginia Tech, John finished a residency program in small animal internal medicine, and is currently satisfying requirements to complete a second residency in clinical neurology. In July of 2001, he became a Diplomate of the American College of Veterinary Internal Medicine, subspecialty of internal medicine, and is planning to sit for the neurology certifying examination in June of 2003. In July of 2003, John will join the faculty of the Virginia-Maryland Regional College of Veterinary Medicine in the Department of Small Animal Clinical Sciences.